(Pages 255 to 354)

Owing to an error in pagination of issue No. 6,
these pages were omitted.

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Trends and Cycles in Surface Temperatures of the Canadian Atlantic

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ABSTRACT

Temperature variations in the surface sea water at St. Andrews, N.B., indicative of variations in surface water temperatures over the main portion of the Atlantic coast of North America, have been expressed mathematically, and cycles of 3.3, 9 and 15 years are involved. It is suggested that cyclic variations in tidal forces and sun spot activity are associated with the variations in the surface water temperatures.

INTRODUCTION

Surface water temperatures have been determined twice daily, over a period of years, at a number of stations on the Atlantic coast of Canada (Hachey 1939). In particular, at St. Andrews, N.B., these observations have been continuous since 1921.

Due to strong tidal action in the bay of Fundy, the water temperatures at St. Andrews, N.B., not only reflect the water conditions in the bay of Fundy, but are indicative, as well, of the general surface water temperature situation over the Canadian Atlantic coast (Hachey and McLellan 1948).

Monthly and Seasonal Mean Temperatures

The monthly mean temperatures for the years 1921 to 1947, for St. Andrews, N.B., are furnished in table I, and the seasonal mean temperatures for the same period are plotted in figure 1. Monthly mean temperatures varied from a low of −1.7°C. in January, 1923, to a high of 14.7°C. in August, 1947. Thus these extremes of the monthly mean temperatures were observed within a period of twenty-four years.

A cursory examination of the plotted seasonal mean water temperatures in figure 1 suggests that sub-normal water temperatures prevailed in the periods 1922 to 1926, and 1939 to 1943, and that supra-normal temperatures prevailed in the periods 1928 to 1932, and 1944 to 1947. There is, therefore, indicated the possibility that there is a cycle including approximately four years of sub-normal temperatures and approximately four years of supra-normal temperatures. This is further borne out in that it is noticeable that a transition from an abnormally cold year to an abnormally warm one usually took place over a number of years, there being only three instances of an abrupt change from an abnormally cold

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to an abnormally warm year or vice versa. Short term trends are therefore indicated in the gradual tendency towards warmer waters over a short period of years such as 1934 to 1937, or the general tendency towards colder waters over a period of years such as 1921 to 1923. Long-term trends, on the other hand, are indicated by the general tendency towards warmer summer temperatures, which within the period 1921 to 1947 have culminated in the consistently higher monthly mean water temperatures at St. Andrews throughout the months of July, August, September and October (Hachey and McLellan 1948).

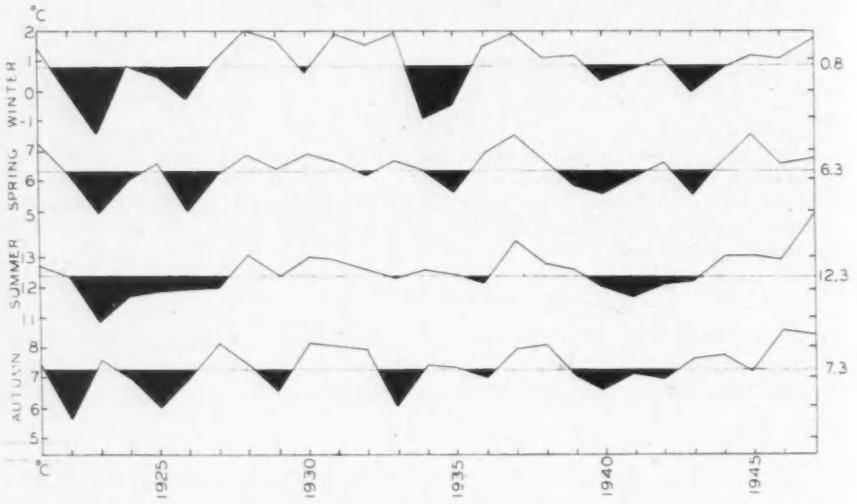

FIGURE 1. Seasonal mean surface-water temperatures at St. Andrews, N.B.

TRENDS

When surface water temperatures are higher than normal, there is a tendency to look for the explanation in the immediate higher incident solar radiation, with warming of the waters to an unusual degree. Similarly, when surface water temperatures are sub-normal, lower incident radiation, or the after effects of winter cooling, are sought as an explanation. In table II increments in monthly mean surface water temperatures for four years, representing abnormally warm (1937, 1947) and abnormally cold surface water temperatures (1922, 1940) are summarized. The rate of vernal warming, as determined by the increase in the monthly mean surface water temperatures from April to July, varied from 8.5 to 9.7 degrees Centigrade, being lowest for 1937, a year of abnormally high surface water temperatures. The warming from July to August varied from 0.4 to 1.8 degrees. In 1940, a year of sub-normal surface water temperatures and annual mean of 6.1°C., the July to August warming was 0.9 degrees Centigrade. In 1947, a year in which were observed the highest surface water temperatures of the period 1921 to 1947, and an annual mean of 7.8°C., the July to August

TABLE I. Monthly mean surface-water temperatures—St. Andrews, N.B.

Year	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Mean
	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.
1921	1.8	0.3	1.8	4.2	7.7	9.6	12.0	12.9	13.0	11.2	6.8	3.2	7.0
1922	−0.2	−0.5	0.5	3.2	6.3	9.1	12.2	12.6	12.2	9.4	5.7	1.7	6.0
1923	−1.2	−1.7	−1.7	1.0	5.7	7.6	10.2	10.9	11.4	10.0	7.5	5.3	5.4
1924	1.6	−0.3	1.2	3.5	6.4	8.2	11.1	12.2	11.9	10.3	7.3	3.3	6.4
1925	0.1	0.0	1.5	3.7	6.9	8.9	11.4	12.3	11.7	8.6	6.3	3.1	6.2
1926	0.5	−1.1	−0.3	1.6	5.3	8.0	11.2	12.2	12.1	10.4	7.4	3.0	5.9
1927	1.6	0.3	1.3	3.4	6.2	9.1	11.4	12.4	11.8	10.8	8.6	5.0	6.8
1928	2.7	1.4	1.8	3.8	6.8	9.9	13.0	13.5	12.4	10.6	7.0	4.9	7.3
1929	2.2	1.3	1.7	3.1	6.8	9.4	11.7	12.6	12.5	10.2	7.1	2.3	6.7
1930	1.3	−0.3	0.7	3.3	6.8	10.2	12.3	13.2	13.2	11.3	8.4	4.6	7.1
1931	2.9	1.0	1.7	3.8	6.6	9.3	12.4	13.4	12.7	10.8	8.6	4.7	7.3
1932	3.0	0.7	0.7	3.3	6.3	9.0	11.4	13.1	12.9	11.4	7.6	4.3	7.0
1933	2.6	1.5	1.4	3.7	6.9	9.6	11.8	12.7	12.3	10.5	6.0	1.6	6.7
1934	−0.4	−1.4	−0.5	3.4	6.5	9.1	11.6	12.7	13.1	10.4	7.6	3.3	6.3
1935	0.0	−0.8	−0.4	2.5	5.3	9.3	12.0	13.1	12.0	10.1	8.0	3.7	6.2
1936	1.4	−0.2	1.9	3.6	6.5	10.4	11.3	12.8	12.2	10.4	7.0	3.7	6.8
1937	2.6	1.3	1.5	4.1	8.1	10.3	12.6	14.4	13.5	10.8	8.0	4.6	7.7
1938	1.7	0.7	0.9	3.8	6.7	9.7	12.0	13.6	12.4	10.9	8.5	4.8	7.1
1939	2.3	0.6	0.6	2.5	6.2	8.7	11.9	13.2	12.4	10.4	6.7	3.9	6.6
1940	0.7	−0.2	0.3	2.3	5.8	8.6	11.5	12.4	11.8	9.4	7.0	3.5	6.1
1941	1.2	0.4	0.4	3.4	6.2	8.8	11.4	12.1	11.5	9.9	7.4	4.0	6.4
1942	1.6	0.0	1.3	3.6	6.5	9.7	11.2	12.5	12.2	10.4	7.5	2.8	6.6
1943	−0.1	−0.4	−0.1	2.1	6.0	8.5	11.5	12.9	12.3	10.7	8.0	4.2	6.3
1944	1.9	0.0	0.3	2.8	6.8	9.1	12.0	13.4	13.3	10.8	7.7	4.5	6.9
1945	0.8	0.3	2.1	5.1	7.6	9.9	12.8	13.8	12.2	10.6	7.5	3.5	7.3
1946	1.0	0.5	1.5	3.6	7.0	9.0	12.1	13.3	12.9	11.8	8.8	4.9	7.2
1947	1.6	1.2	2.1	3.6	6.7	9.9	13.7	14.8	14.2	12.0	8.8	4.3	7.8
Mean	1.3	0.2	0.9	3.3	6.5	9.2	11.8	12.9	12.5	10.5	7.5	3.7	

TABLE II. Mean surface-water temperature increments for typically abnormal years.

Year	Abnormality	Increment April to July (degrees)	Increment July to August (degrees)
1922	cold	9.0	0.4
1937	warm	8.5	1.8
1940	cold	9.2	0.9
1947	warm	9.7	1.1

warming was only 1.1 degrees. Hence we must look for some cumulative effect, or trend, in order to explain the observed yearly variations in the monthly mean surface water temperatures.

Particulars of surface water temperatures at St. Andrews, N.B., for the years preceding abnormally cold (1923 and 1940) and abnormally warm (1937 and 1947) surface water temperatures are furnished in table III. The colder monthly mean surface waters of 1923 are noted as following from a steady downward trend in the annual mean, the annual maximum, and the annual minimum temperatures in the period 1921 to 1923. A similar trend is noted for the period 1937 to

TABLE III. Particulars of surface-water temperatures at St. Andrews, N.B., for the years preceding abnormally cold (1923 and 1940) and abnormally warm (1937 and 1947) years.

Year	Mean annual temperature (°C.)	Maximum monthly temperature (°C.)	Minimum monthly temperature (°C.)
1921	7.0	13.0	0.3
1922	6.0	12.6	−0.5
1923	5.4	11.4	−1.7
1934	6.3	13.1	−1.4
1935	6.2	13.1	−0.8
1936	6.8	12.8	−0.2
1937	7.7	14.4	1.3
1937	7.7	14.4	1.3
1938	7.1	13.6	0.7
1939	6.6	13.2	0.6
1940	6.1	12.4	−0.2
1944	6.9	13.4	0.0
1945	7.3	13.8	0.3
1946	7.2	13.3	0.5
1947	7.8	14.8	1.2

1940 leading to the abnormally low mean surface water temperatures of 1940. The warmer monthly mean surface water temperatures of 1937 are noted as following from a generally upward trend in the annual mean, the annual maximum, and the annual minimum temperatures in the period 1934 to 1937. A similar trend is noted for the period 1944 to 1947 leading up to the abnormally high mean surface water temperatures of 1947. Short term trends would therefore seem to be well established.

PERIODICITY AND LONG TERM TRENDS

Using the surface water temperature data at St. Andrews, N.B., a search for

systematic variations of a cyclic nature, culminated in the determination of the following equation:

$$t = 6.7 + 0.5 \sin\left[\frac{2\pi(y-20.0)}{3.3}\right] + 0.6 \sin\left[\frac{2\pi(y-17.5)}{9}\right] + 0.3 \sin\left[\frac{2\pi(y-13.3)}{15}\right]$$

where

t = annual mean surface water temperature at St. Andrews, N.B., in degrees Centigrade

y = the year number, e.g., 43 for 1943.

The observed annual mean surface water temperatures at St. Andrews, N.B., are plotted in figure 2(a), and superimposed upon the curve represented by the above equation. A fair agreement is obvious, and the correlation coefficient by cross-products is greater than 0.9 (Brunt 1931).

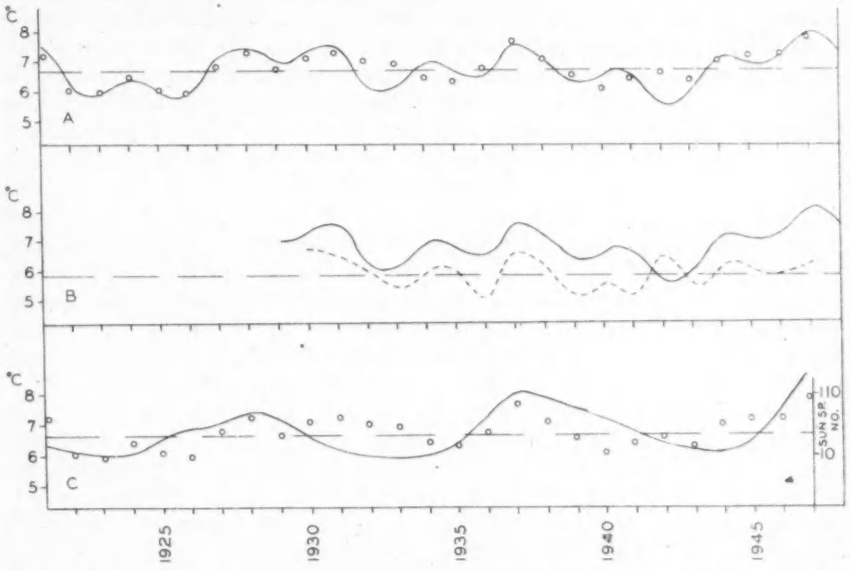

FIGURE 2 (A) Annual mean surface-water temperatures at St. Andrews, N.B., superimposed upon the curve for the equation:

$$t = 6.7 + 0.5 \sin\frac{2\pi(y-20.0)}{3.3} + 0.6 \sin\frac{2\pi(y-17.5)}{9} + 0.3 \sin\frac{2\pi(y-13.3)}{15}$$

(B) Annual mean surface-water temperatures at Entry island, P.Q., in relation to the curve for the equation representing St. Andrews temperatures.

(C) Annual mean surface-water temperatures at St. Andrews, N.B., in relation to sun spot numbers.

The method of determining the equation was a trial and error method, the shorter periods being suggested by the plotted data, the final term being required because of the suggested long term trend, or general tendency toward higher

monthly mean water temperatures throughout the period 1921 to 1947 (Hachey and McLellan 1948). A search for periodicity by statistical treatment of the temperature data (Whittaker and Robinson 1924), gives a periodogram with peaks at 2.5, 3.4, 4.5, 6.2 and 9 years. The more prominent peaks of the periodogram were at 2.5, 4.5 and 9 years. While this result tends to give some substance to periodic values of 2.5 and 4.5, the data are even too scant to give any reliability to the 9 year value. The value of the equation must, therefore, rest upon its ability to fit the data. With the exception of the years 1932 to 1935 and 1942, the fit is satisfactory.

APPLICABILITY TO OTHER ATLANTIC POINTS

Surface water temperatures have been determined twice daily, except during the winter months, at Entry island of the Magdalen islands in the gulf of St. Lawrence since 1930 (Hachey 1939). Allowing a minimum temperature of −1.8°C. for the winter months (the approximate freezing point of the surface waters of the gulf of St. Lawrence), annual mean surface water temperatures for Entry island were calculated, and are plotted in figure 2(b) in relation to the theoretical curve as determined for St. Andrews, N.B. Upon inspection, the

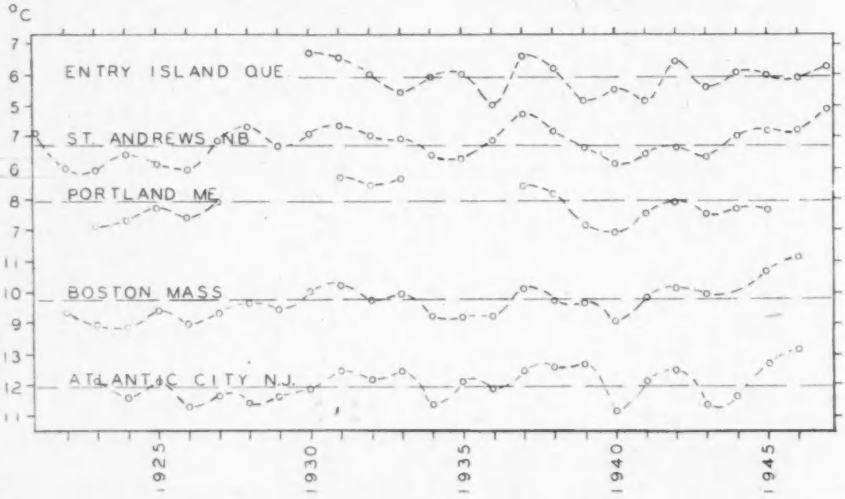

FIGURE 3. Annual mean surface-water temperatures for various points on the Atlantic coast of North America.

general cyclic features expressed by the equation are in evidence in gulf of St. Lawrence data. One interesting feature is that where St. Andrews annual mean surface water temperatures for the years 1932, 1933, 1940, 1942, fail to fit the theoretical curve and fail to follow the general trend, the corresponding data for Entry island do.

In figure 3, annual mean surface water temperatures at Portland, Me.,

Boston, Mass., and Atlantic City, N.J., (U.S. Department of Commerce 1947) are plotted in comparison with the corresponding surface water temperatures at St. Andrews, N.B., and Entry island, P.Q., and it becomes obvious that the outstanding cycles and trends as exhibited in the surface water temperatures at St. Andrews, N.B., are common to the waters of the main portion of the Atlantic coast of North America.

DISCUSSION

The foregoing analysis is suggestive of cycles of surface water temperatures, involving periods of 3.3, 9, and 15 years. The 3.3 and 9 year periods have some significance, as cyclic phenomena in nature, involving periods of similar magnitude, have been dealt with by numerous authors.

One of the principal components of the tide-producing forces can be derived from the angular velocity of the movement of the long axis of the elliptic orbit of the moon, which makes one complete rotation in 8.85 years (Sverdrup *et al.* 1942). This period of 8.85 years is involved in the theory of "internal waves" (Petterson 1930), the principles of which form the basis of the theory of transgressions which are defined (Le Danois 1934) as a "periodic movement, of variable amplitude, of Atlantic waters of tropical origin, bringing a momentary encroachment of these waters upon the waters of polar origin and especially upon the continental waters". Involved also in the cyclic variation of astronomical tidal forces is a period of 4½ years. There is, however, on the basis of our own observations no apparent direct relation between transgressions and surface water temperatures (Hachey 1936). It is, in any event, reasonable to assume that periodic variations in the tidal forces, would, under certain circumstances, be reflected in the observed surface water temperatures, but particularly in an area where tidal mixing of the water layers is a large scale phenomenon (Hachey 1934.)

Sun spot activity, which has been evoked as a possible explanation of so many terrestrial phenomena, is considered cyclic in character. In figure 2(c), the observed annual mean surface water temperatures at St. Andrews, N.B., are superimposed upon a curve representing the annual variation in sun spot numbers, in the period 1921 to 1947 (Stetson 1947). In the period 1921 to 1947, peaks of sun spot activity were experienced in 1928, 1937, and 1947, giving an average cycle of 9.5 years, and coinciding with the observed peaks in the annual mean surface water temperatures. Many hypotheses have been put forward to explain observed relationships between the weather and the sun. It has, however, been observed that "with sunspots below some critical value, temperatures on the earth seem to go up with increasing sun spots" (Stetson, p. 112, 1937). Tannehill (1947) presents evidence to support the conclusion that when the circulation of the atmosphere becomes more vigorous because of changes in solar radiation, the winds tend to lower the temperature of the ocean surface by mixing.

SUMMARY

1. Seasonal and annual mean water temperatures at St. Andrews, N.B., exhibit short and long term trends.

2. An equation has been derived, involving cycles of 3.3, 9, and 15 years,

which approximates the annual mean surface water temperatures at St. Andrews, N.B., which temperatures are generally indicative of variations in the surface water conditions over the main portion of the Atlantic coast of North America.

3. The 3.3 year cycle only roughly approximates a 4.5 year cyclic variation of astronomical tidal forces. The 9 year cycle, during the period 1921 to 1947, corresponds with a tidal cycle of 8.85 years, and also with the frequency of sun spot peak numbers of recent years.

4. It is reasonable to assume that variations in both the strength of astronomical tidal forces and sun spot activity would be reflected in variations in surface water temperatures.

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Odontosyllis at Bermuda and Lunar Periodicity

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ABSTRACT

At Bermuda, the polychaete worm, *Odontosyllis enopla*, approaches the surface and lumin-esces in connection with spawning monthly after full moon, beginning about 55 minutes after sunset and lasting half an hour. In mid-winter this display was correlated with no moon at that time after sunset irrespective of clouds which did not seem to affect visibility. Response only to decrease in light intensity from sunlight to starlight gives lunar periodicity. There is similar behaviour in other species of *Odontosyllis* and also in *Platynereis* and *Nereis*. Apart from spawning, the prawns *Anchistioides* and *Penaeopsis* swarm at the surface in similar fashion, and salmon parr approach the surface most nearly when sunlight decreases to starlight.

The recent paper by Korringa (1947), reviewing "relations between the moon and periodicity in the breeding of marine animals", shows that unpublished conclusions as to the mechanism for lunar periodicity in the spawning of the annelid worm, *Odontosyllis enopla* Verrill, as observed at Bermuda in 1926, are still new. They seem to solve, for this species at least, the puzzle of lunar periodicity. Starting with observations on the European oyster, Korringa has surveyed the various instances on record and concludes that the lunar periodicity is "called forth by the sequence of neaps and springs in those regions where the tidal range is considerable" and that "with little or no tidal amplitude other factors could be held responsible . . . in nocturnal animals, sensitive to light, the alternation of dark and moonlight nights appeared to be closely related with sexual maturation". The Bermudan species reveals a mechanism for spawning under the latter conditions, but not that sexual maturation is determined by the moon.

Galloway and Welch (1911) investigated *Odontosyllis enopla* in 1904 at the Flatts inlet, Bermuda. Spawning individuals were observed July 3-7, 29-31 and August 23, giving a lunar rhythm. They appeared "within fifteen minutes of the same time, just as dusk was becoming pronounced" and "the display lasted from twenty to thirty minutes". The larger females appeared near the surface and luminesced over the whole body leaving luminous trails as they swam in circles of two inches (1 in. = 2.5 cm.) or more in diameter. The males rose toward them, giving sharp intermittent flashes, and when a male reached a female, they rotated together in somewhat wider circles.

The late Dr. E. L. Mark, who maintained a connection with, and a very active interest in, the Bermuda Biological Station until his death about two years

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ago at an advanced age, told me of the spawning behaviour of this worm which is well known in Bermuda as the "fire fish". He stated that the behaviour was to be observed for several nights after the full moon, and in particular that it had a most precise relation to light. One could set one's watch within five minutes of the correct time by observing the first appearance of the worms in the evening and adding 50 to 55 minutes to the published time of sunset for the day! I expected that he would publish his observations, but he did not do so. Also, I hoped to be able to visit Bermuda again and make further and more detailed observations including precise measurements of light intensity, but this has not yet been possible.

From January 21 to March 9, 1926, I lived at the Grasmere Hotel, Pembroke, Bermuda, owing to its proximity to Agar's island, on which the Biological Station was then located. The hotel is at the head of a deep narrow inlet, about half a mile long on the east side of Great Sound, which leads to Hamilton harbour. The worms could be observed off the boat landing below the hotel. It was a great advantage that the spawning evolutions could be witnessed in the dark without the use of artificial light, which might have affected the behaviour. It should be made clear that it was the display of luminescing worms that was actually observed. No attempt was made to determine that the worms were sexually mature and that they actually spawned. It is spawning behaviour as having been described as related to spawning.

The worms live on the bottom. Those captured and put in a pail or bottle went to the bottom and into the angle with the side, where each, sometimes within 24 hours, constructed a tube of webbing, somewhat longer than itself, open at each end, and lay with the head just protruding from one opening. Observations on 12 evenings as to where the worms first appeared gave on the whole earlier appearance in shallow water and at the head of the inlet. They were observed from close to shore out to depths of 3 fathoms or more. A fisherman informed me that he had seen them far from land over depths as great as 10 fathoms (1 fathom = 1.83 m.).

A fisherman stated that he knew the worms only in summer and that they were eaten in quantities by certain of the fishes, which, when seined the next morning, were found to be gorged with the worms. On the evening of March 6, when the worms were fairly numerous, fish were seen jumping at the water surface and seemed to be catching the worms as they glowed, as there were repeated jumps at one point where the luminescence was and the light seemed to jump out of the water repeatedly as if moved by the fish.

When a female comes to the surface, she glows for 3 to 8 seconds and this may be repeated as many as 33 times and over a period of 12 minutes, but sometimes only one glow is seen. They occur at intervals of from 10 to 50 seconds, but may follow each other so closely that the interval appears only as a lessening in intensity. Sometimes no males appeared even when a flashlight was used to attract them and with a large number of females glowing from 1 to 7 times each (February 8). When males appeared quickly (March 6), the females did not

seem to glow more than 3 times each, if so many, but, even when glowing from 3 to 7 or more times (March 7), two males were seen to approach one female simultaneously. Each male gave 2 or 3 quick flashes on its way to the female and its flashes were distinguishable in the subsequent combined nuptial circling. The circles of each female at first were about 2 in. in diameter and increased to about 6 in. in diameter after she was joined by a male. Females were observed, both in the open and when put in a pail, going spirally to the bottom after the last glow. In a pail, they might resume glowing repeatedly for some time in midwater before going to the bottom.

Mr. Edwin B. Damon, who was carrying on physiological investigations in a laboratory in the hotel, kindly gave me records of his observations of display of luminescence by *Odontosyllis* in this inlet for the previous four months or so, starting in October of 1925. The numbers of the *days after full moon* on which he actually observed them in each month were as follows: October, 1 to 8; November, 3 to 8; December, 4 to 10; January, 6 to 13; and February 4 to 8+. The length of time after sunset at which the worms first appeared on the 33 days, ranged from 51 to 62 minutes, with an average of $55\frac{3}{4}$ min. To some extent in October and to a marked extent in November, with full development of the spawning behaviour, males were more abundant than females, even to 4 or 5 times, and there might be a large number of males showing before any females appeared. Early and late in the monthly period, with few worms, there might be no males. By February, males were few or absent.

My own observations were from February 3 to March 7 inclusive. The display was observed from February 3 to 12 (6th to 17th day after full moon) and from February 28 to March 7 (1st to 8th day after full moon). The length of time after sunset at which the worms first appeared on 13 days of observation ranged from 53 to 60 minutes, with an average of $56\frac{1}{2}$, but, for lack of a reliable chronometer, these figures are not strictly dependable. In February (3 to 12) only one male was certainly observed and when females were most numerous, not a single male was discovered by using a flashlight to attract them. In March, there were no males at first, but they were found first by using a flashlight (3), then noticed without it (Mar. 4 and 5), and finally (Mar. 6) appeared quickly when the female display commenced. They were never very numerous.

When the worms were reasonably numerous, the display lasted from 13 to 25 minutes. In February, the numbers were very low during the last four days of the moonless period. Starting the next moonless period on the last day of February, the numbers built up steadily on successive days, omitting March 2, when none were seen, as follows: 1, 3, 7, 12+, ca. 36; and they remained high to the last day of observation (Mar. 7).

In determining the exact time of the appearance of the worms, it was soon apparent that, as the time approached, it began to be impossible for me to read the time on my watch without using a flashlight or lighting a match. However, when the worms ceased to appear, starting with February 13, it was possible to read the time because the moon was then above the horizon. During the period

of my stay, there were 15 days when the moon was up at 55 minutes after sunset, on 11 of these I made observations and on no occasion was there any display with or without overcast sky. There were 18 days when the moon was not up at 55 minutes after sunset, on 17 of these observations were made, and on 15 a display was observed. It is to be noted that, even when the sky was overcast, I could read the time when the moon was up. Also, on one of the nights (March 2) when the moon was not up and no worms appeared, I was able to read the time more than an hour after sunset, whatever may have been the reason. Accurate measurements of light intensity would have been valuable.

It would seem that, even with fewer individuals maturing at the low temperature of midwinter, and with a paucity of males, the spawning period for the month is longer than with high temperature in summer. Low temperature doubtless slows down the whole process of maturation and voiding of the sexual products. This lengthening of the period in winter permits the appearance of a very definite correlation between spawning and the absence of moonlight at about 55 minutes after sunset. It seems reasonable to conclude that, for this species at least, which lives in tubes at moderate depths, a spawning migration to the surface is elicited in those individuals, which are sufficiently mature, by a drop in light intensity from daylight to starlight, but not from daylight to moonlight. From new moon to full moon, the spawning reaction fails to be elicited. The many individuals that mature during this period at high summer temperatures will spawn somewhat precipitately when they begin after full moon to be exposed every evening to a drop in light intensity from sunlight to starlight. It appears that, at least during the winter, the stimulus needs to be repeated for several days before most worms respond.

DISCUSSION

It is evident that *Odontosyllis enopla* at Bermuda presents an exceptionally favourable opportunity for elucidation of a mechanism of lunar periodicity in spawning. The tides are too slight to have any particular complicating effect. Both sexes luminesce, making their presence noticeable. The display occupies a very short period at a time of day that is convenient for observation. Also, the worms live in very shallow water, where the conditions are little different from those at the surface where observations are made. This is important, since the phenomenon is a zoapocritic one (Huntsman 1948), that is, it is a response of the animal as a whole to what it faces where it lives. For elucidation, the dynamic conditions that elicit the response where the animal is living must be discovered.

To what extent there may be a similar mechanism in other instances of lunar periodicity that have been described, is open to question. More detailed observations than have yet been made seem to be required. *Odontosyllis phosphorea* Moore, on the coast of British Columbia, also spawns in relation to sunset (Potts 1913; Fraser 1915). Spawning has been established as "definite as regards the position of the sun, not definite as to time on the clock", and to be "from sunset

or possibly a little before it until almost dusk, that is for a period lasting from half an hour to an hour" (Fraser). There seems to be no approximation of the sexes, perhaps because there is no luminescence. The worms circle with a "wavy wiggle". The earlier appearance in relation to sunset may be associated with the fact that in relation to Departure bay, where the observations were made, the worms were found on the bottom just outside the bay at depths of from 30 to 90 fathoms inside the debris of dead Hexactinellid sponges (Potts). In this situation, decrease in light intensity at sunset would be earlier than in shallow water. Although the spawning was at first thought to be at a particular time of the year and of the lunar cycle, more extended observations showed that it occurred at least from as early as July to as late as December and also in all four quarters of the lunar cycle. However, the data give some indication that spawning is most abundant for eight days following the full moon and at times equally abundant at some time during the ten days beginning with new moon.

Odontosyllis hyalina Grube, in the bay of Batavia living in shallow places above the reefs, has been found by van Lummel (1932) according to Korringa (1947), to spawn all the year round, swarming after sunset during the three nights following the full moon. They were found to be very sensitive to light. This behaviour is quite similar to that of the Bermudan species.

Platynereis megalops (Verrill) has been found by Just (1914) and *Nereis limbata* Ehlers by Lillie and Just (1913), both at Woods Hole, Massachusetts, to spawn in swarms in the summer during an hour or two, starting just after twilight and between full moon and new moon. This seems to be behaviour somewhat comparable with that of *Odontosyllis*.

The Palolo worm (*Eunice*) behaves very differently in spawning. Only the hind part comes to the surface and this happens but once a year, in spring or early summer. While it is related to the lunar cycle, occurring usually close to the last quarter of the moon, it does not seem to be elicited by light. While the spawning is generally about sunrise, Friedlaender (1898) states that at the Samoan islands it starts before there is any light at all from the sun in the east at 4.00 a.m., both in moonlight and with heavily clouded sky, and in other places soon after midnight and with well advanced dawn. Gravier (1924) reports that these worms spawn from 9.00 to 11.00 p.m. at New Hebrides, and this would be in starlight. He also mentions that these or similar worms are taken in full daylight at certain places in those islands. Such spawning is not at all comparable with that of *Odontosyllis*.

While the behaviour described has been considered to be associated with spawning, it is not certain that this is invariable. When females alone appear, the eggs will not be fertilized and may not be voided. The matter requires investigation. A lunar periodicity in coming to the surface without reference to spawning has been observed at Bermuda by Wheeler (1937) in the prawns *Anchistioides* and *Penaeopsis*. This behaviour seems to be rather closely similar to that of *Odontosyllis* in coming to the surface, since "usually it begins about an hour after sunset, and in consequence the period is later each month from December

to July and earlier thereafter" and also "it takes place on clear starlit nights, during the appropriate period of the lunar cycle and does not occur at other times when the light of the moon is totally obscured" (Wheeler 1937).

Some fishes come near or to the surface about an hour after sunset, and then seem to become rather generally distributed through the remainder of the night. I have observed salmon parr to come close inshore at that time and, if there was no moonlight when twilight had gone, they came to the immediate surface so as to make a splashing noise when disturbed.

There is particular need to relate this rather general behaviour to the actual conditions of light intensity. Changes in light intensity should be followed by measurement. Johnson (1938) found that the copepod *Acartia clausi* comes to the surface after sunset and also in laboratory experiments moves to the surface when the light intensity is decreased. That clouds do not alter the behaviour does not necessarily rule out moonlight as the factor responsible for the periodicity.

SUMMARY

Odontosyllis enopla at Bermuda comes near the surface and luminesces in connection with spawning during a half-hour interval, beginning about 55 minutes after sunset. This occurs each month after full moon. In mid-winter, this display is at a low ebb and females predominate. At that time the display was found to be correlated with no moon after sunset irrespective of clouds, which did not seem to affect one's seeing the time on a watch. It is concluded that this response of the worms, which live in woven tubes on the bottom in crevices in shallow water, is to rapid decrease in light intensity to starlight, but not to moonlight. As a result of this, there is lunar periodicity in the appearance and the spawning of the worms.

Other species of *Odontosyllis* on the coast of British Columbia and in Java behave somewhat similarly in relation to light. Species of *Platynereis* and *Nereis* on the coast of Massachusetts spawn with less precise relation to sunset and absence of moon.

The spawning of the Palolo worm (*Eunice*), although occurring in relation to the lunar cycle (once a year, in the moon's last quarter), gives no evidence of being elicited by light.

Apart from spawning, the phenomenon of coming to the surface when sunlight decreases to starlight rather than moonlight has been observed in the prawns *Anchistio les* and *Penaeopsis*. Also fish, such as salmon parr in freshwater streams, sometimes approach the surface most nearly when sunlight decreases to starlight.

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Bacteria Associated with Spoilage of Cod Fillets

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ABSTRACT

When sterile cod muscle was inoculated with pure cultures of bacteria and held at 3°C., it was found that the common spoilage odours were rapidly produced by *Pseudomonas*, *Proteus*, *Achromobacter* and *Serratia* species. *Micrococcus* and *Flavobacterium* species produced milder forms of spoilage and much more slowly. Mesophilic species which rapidly decompose fish at 25°C. produce no apparent spoilage at 3°C. even when the initial inoculum contains a very large number of cells.

Under suitable environmental conditions fish muscle will support the growth of a very wide range of bacteria. The muscle itself, or a digest or extract of the muscle, can be used as the source of nutrients in culture media and will produce luxurious growth with many common saprophytes. This would lead one to believe that fresh fish spoilage may ordinarily result from the activity of a great many different types of bacteria.

Such a supposition is based on the premise 'under suitable conditions'. However, in commercial practice these suitable conditions for many bacteria do not exist. From shortly after they are caught until shortly before they are eaten, fresh fish are held on ice or in refrigerators near freezing temperatures. At blood heat or room temperature, fresh fish spoil so rapidly that soon they are inedible. By far the greatest part of the spoilage in commercial fresh fillets occurs while the fish are stored at low temperatures (0 to 5°C.).

Furthermore, the organisms causing spoilage must necessarily be limited to those which normally contaminate the fillets during some stage of their handling,

Many surveys have been made to determine the microflora in the slime. gills and faeces of fish (see Griffiths 1937; Dyer 1947). Similar surveys have been made of sea water, ice and other sources of contamination with which the fish come in contact. These combined efforts have given us a reasonably clear picture of the initial microflora on fresh fillets and of the source of the significant genera. But to a great extent the picture stops there. Which of all the organisms on the surface of a freshly cut fillet are those that will later cause it to spoil? Is any particular group, genus or species significantly more important than any others? Do all the organisms that are present continue to grow, or is there some selective action resulting in a proportional increase in some groups and a decrease in others? As fish spoil they develop a succession of odours and flavours. Are these the

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result of successive groups of bacteria, or successive changes in the fish, brought about by a single group of bacteria?

An attempt has been made to answer some of these questions, and particularly to determine what types of bacteria are responsible for normal fresh cod fillet spoilage.

Spoilage Potential of Species

In attempting to classify the fish-spoiling potential of bacteria, we must first determine by what criterion we can judge whether an organism will play a significant part in spoilage. The mere fact that it reduces trimethylamine oxide, or decomposes protein, or hydrolyses fat, or produces hydrogen sulphide in culture media specifically prepared for these tests, does not necessarily mean that it will grow and bring about a similar change in fish held at a much lower temperature (table I). To be significantly harmful, an organism (1) must be able to *grow* on fish at the temperatures at which fillets are usually held, and (2) must produce some objectionable change in the colour, flavour, odour or texture of the fish.

Table I. Various reactions in culture media under standard conditions at 25°C., with the development of odours resulting from a heavy inoculation of these same organisms on codfish muscle at 3°C. after 20 days' storage.

	Culture media incubated at 25°C.				Cod fillets incubated at 3°C.
Organism	Proteolysis	Trimethylamine oxide reduction	H_2S production	Indol production	Odour
Escherichia coli	−	+	−	+	none
Aerobacter aerogenes	−	+	−	−	none
Bacillus subtilis	+	−	+	−	none
Bacillus mycoides	+	−	−	−	none
Micrococcus citreus	−	−	−	−	none
Micrococcus aureus	+?	−	+	−	none
Sarcina lutea	+?	−	+	+	none
Flavobacterium marinum	−	−	−	−	sour
Pseudomonas fluorescens	+	−	+?	−	putrid
Proteus vulgaris	+	+	+	+?	putrid
Achromobacter sp.	−	+	−	−	putrid

The ideal procedure would be to use the fish itself as a substrate, to hold it at approximately freezing temperatures, and, after storage, to subject it to a thorough chemical analysis. But this is impractical because of the wide variety of volatile substances which can be formed by different types of bacteria from fish. Trimethylamine is a very useful measure of the early progress of spoilage in fish contaminated with the natural, mixed microflora; but neither this, nor any other single chemical test can be used as a quantitative measure of "fish spoilage"

where pure cultures are concerned. An organism might cause spoilage by the formation of trimethylamine, of indol, or of hydrogen sulphide. It is for this reason that the empirical values obtained by organoleptic tests continue to be of value.

In initial stages of this work the following method was used for determining the spoilage capacity of a bacterial culture: Pieces of similar size from untreated sterile cod fillet were inoculated with decimal dilutions of the bacteria in question and were incubated in covered glass containers held at 3°C. Daily, for twenty days, two independent observers examined tne fish for the development of spoilage, as well as for any observable changes in the colour or texture of the fish. At the time of inoculation, plate counts were made to determine the approximate numbers of bacteria in the dilutions used.

Use of Autoclaved Fish Media

The most serious objection to the use of fresh untreated fish muscle as a substrate for testing bacteria was the difficulty in obtaining the sterile muscle, and the cumbersome technique where large numbers of tests were involved. To

Table II. Time required for the development of definite spoilage odours on fresh fish muscle and autoclaved fish media with and without agar, by pure cultures of bacteria.

Culture (no.)	Genus	Cells of inoculum (no. per c.c.)	Fresh fish	Fish medium without agar	Fish medium with agar
			Days	Days	Days
416	*Pseudomonas*	6×10^6	6	6	6
"	"	34×10^2	8	9	9
180	"	20×10^6	6	6	6
"	"	12×10^2	9	9	8
183	"	148×10^6	5	6	5
"	"	2×10^6	6	6	6
"	"	2×10^2	13	14	13
55	*Flavobacterium*	13×10^6	10	10	10
"	"	17×10^2	−*	−*	−*
271	*Proteus*	29×10^6	5	5	6
"	"	13×10^6	7	7	7
"	"	26×10^2	7	7	8
257	*Achromobacter*	280×10^6	5	5	5
"	"	10×10^6	6	6	6
"	"	11×10^2	10	9	10
169	*Micrococcus*	1×10^6	12	11	12
"	"	1×10^2	−*	−*	−*
3	"	13×10^6	13	11	12
41	"	3×10^6	14	13	14
5	"	27×10^6	10	11	11

*No odours developed up to 14 days.

overcome this difficulty, various methods of sterilizing the fish were tried, including treatment with ethylene oxide, intermittent heating and autoclaving for different periods of time. It was subsequently found that an autoclaved fish medium composed of equal weights of finely macerated fresh cod muscle and water, with or without the addition of agar to 0.05%, could be used in place of the untreated fresh fish. Approximately equal surface areas of fresh fish muscle and fish media were inoculated with similar dilutions of various bacterial suspensions. These were incubated at 3°C. and examined daily for the development of spoilage odours. Tables II and III show the result of 28 such tests, using both pure cultures and natural mixed contaminants. The chief advantage of adding 0.05% of agar to the fish is that it facilitates observations on the development of the bacteria on the surface of the medium.

Although freshly incised sterile fish muscle was used to some extent throughout all the experiments which follow, the greater proportion of the results were obtained with the fish-agar medium, prepared in ordinary petri dishes.

TABLE III. Number of days required to develop spoilage odours from fish muscle and fish media inoculated with fish slime, water from melted ice and mixed cultures from fillets.

Source of contamination	Bacteria (approx. no. per ml.)	Fresh fish	Fish medium without agar	Fish medium with agar
		Days	Days	Days
Fish slime	120×10^6	1	1	1
" "	120×10^5	10	11	11
Dirty ice	100×10^6	6	6	6
" "	100×10^5	9	10	10
Clean ice	4×10^2	8	9	8
Fillets	100×10^6	7	7	8
"	1×10^6	9	9	9
"	2×10^3	12	11	12

SPOILAGE FROM STOCK CULTURES

In our stock culture collection there are 97 strains of identified common saprophytes, any one of which might during some stage of handling become a contaminant on fish fillets. Four graded suspensions were made from each of these cultures, containing from less than 100 to several million cells per ml. One ml. portions of each suspension were spread evenly over approximately the surface of 60 sq. cm. of fish muscle or fish medium and incubated at 3°C. These were observed daily for 20 days.

Based on the effect of the bacteria on the odour of the fish, these organisms could be classified into three distinct groups: (1) Those which either did not grow, or, if they did grow, produced no observable change in the odour of the fish; (2) those which produced musty, sour or lactic-acid like odours, but which never

developed into really offensive types of spoilage; and (3) those which grew rapidly on the fish at 3°C. and readily produced various types of putrid and offensive odours. The following list includes the cultures falling into each of these three groups:

1. *No observable change at 3°C.:*

Aerobacter aerogenes	4 strains	*Micrococcus aurantiacus*	2 strains
Aerobacter cloaceae	1 "	*Micrococcus cinnebareus*	1 "
Escherichia coli	3 "	*Micrococcus rubens*	1 "
Proteus vulgaris	3 "	*Micrococcus aureus*	3 "
Escherichia communior	1 "	*Micrococcus citreus*	2 "
Serratia marcescens	1 "	*Micrococcus roseus*	2 "
Bacillus subtilis	4 "	*Sarcina lutea*	1 "
Bacillus mesentericus	1 "	*Micrococcus candicans*	3 "
Bacillus mycoides	2 "	*Micrococcus varians*	2 "

Many of the organisms of this group, especially members of the *Enterobacteriae* and *Bacillaceae*, produced offensive odours in the fish when incubated at 25° or 37°C.; most of the *Micrococci* at higher temperatures developed spoilage similar to that described for the second group at 3°C.

2. *Musty, sour or sweetish odours at 3°C.:*

Flavobacterium marinum	2 strains	*Micrococcus cinnebareus*	1 strains
Flavobacterium solare	2 "	*Micrococcus epidermidus*	1 "
Achromobacter sp.	1 "	*Microbacterium* sp.	15 "
Flavobacterium proteus	1 "	*Micrococcus* sp.	8 "
Micrococcus varians	1 "		

Most of these organisms grew on the fish at 3°C. Changing the incubation temperatures to 25°C. produced more rapid growth but did not make much difference in the type of odours produced.

3. *Rapid production of offensive odours:*

Pseudomonas sp.	18 strains	*Achromobacter candicans*	2 strains
Achromobacter candicans	2 "	*Serratia marcescens*	2 "
Proteus vulgaris	4 "		

Organisms of this group not only produced a much more offensive type of spoilage, but did so very much sooner than those in the second group.

In estimating the spoilage potential of a bacterial culture two things must always be considered: The effect of temperature on the growth rate, and the changes produced by the organism when grown at a suitable temperature. This is shown by contrasting some of the cultures tested under the first and third groups. At 25 and 37°C., many of the gram negative rods listed as "producing no change in the fish" gave at the lower temperature cultures that were quite as

offensive as those of most organisms of the third group. The chief difference is in the inability of the former group to grow on fish at near-freezing temperatures. A suspension of 250,000,000 cells of *E. coli* brought no change to the fish up to 20 days at 3°, while less than 100 cells produced a putrid fish in 24 hours at 37°C.

Cultures Isolated from Fish

It is obvious that under normal conditions, the mesophilic species of bacteria are relatively insignificant in the spoilage of fresh fillets. The results obtained with the pure cultures point strongly to the psychrophilic gram negative rods as being the types chiefly implicated. To substantiate this point further, tests were made with cultures isolated from fillets during various stages of decomposition. These were similarly inoculated onto sterile fish muscle and fish media and observations made on the changes produced at 3°C. The results were almost identical with those obtained with the stock cultures. Gram negative rods with characteristics of the *Pseudomonas*, *Achromobacter* and *Proteus* genera grew rapidly from small inocula and produced a variety of offensive odours. Many cultures, especially of the *Pseudomonas* species, digested the fish muscle, changing it into a semi-liquid mass.

Species of *Micrococcus*, *Flavobacterium*, *Microbacterium* and other unidentified species produced more slowly various types of "sweet", musty and sour odours, and the chromogenic species frequently coloured the fish. As a group these organisms rarely, if ever, digested the fish or produced strong or putrid odours.

Succession of Odours from Pure Cultures

One striking characteristic of the more serious of the fish-spoiling cultures was that they never produced a single typical odour which gradually increased in intensity as storage continued. Instead, there was usually a succession of two, three or four distinctly different odours, each gradually blending into the previous one as spoilage progressed. For example, some of the *Proteus* cultures began with a slightly "fishy" odour, which later became "fruity" and then through a series of very offensive putrid odours. The green fluorescent *Pseudomonas* cultures never produced anything even faintly resembling the "fishy" odour. Their initial odour was almost invariably fruity, quite similar to that produced by *P. fragi* in milk or cream. Occasionally some cultures were found giving an odour characteristic of potatoes stored in a damp cellar. After one or two days these not unpleasant odours gradually changed into very putrid types. Several unidentified *Pseudomonas* cultures, which were initially "fruity", later lost this odour and after 2 weeks or more the fish were almost odourless. By grading the number of cells in a series of inoculations, it was possible to produce simultaneously several different odours from one culture. The very heavily inoculated fish soon reached the putrid stage while those with lesser inoculum were still going through the sweetish or fruity stages.

Similar observations were noted with the other less harmful cultures. The *Microbacteria* initially produce a slightly sour odour. This gradually increases

until the fish has a distinctly sour milk odour. On further holding, the fish becomes almost odourless. Cultures of *Micrococci* were also observed to produce odours which later changed or disappeared altogether.

Apart from these more general changes, many individual cultures were found to have other very characteristic odours. Two cultures of *Flavobacteria marinum* produced odours almost indistinguishable from formaldehyde; a gram negative unidentified rod gave the fish a nut-like odour.

But as these unusual odours were much slower in developing than the typical spoilage types, it would appear that they could have no practical significance.

Changes in pH

As would be supposed, the fish inoculated with those organisms producing the milder sweet, sour or musty odours showed either no change or a decrease in the pH of the fish. Invariably those cultures (*Achromobacter*, *Proteus*, *Pseudomonas*, *Alcaligenes*) which rapidly produced strongly offensive odours, increased the alkalinity of the fish. When a very heavy inoculum was used an increase in pH, as indicated by brome thymol blue, was frequently observed before the first spoilage odours developed. But, when the spoilage developed after the growth of an initially small inoculum, the increase in pH followed the production of off-odours, by one to six days.

DISCUSSION

During these tests two things were continuously looked for but not encountered. The first was a serious type of fillet spoilage resulting from the activity of *Micrococci*. With the stock cultures, as well as with *Micrococci* isolated from fresh fillets and fish slime, the results were almost always the same: Relatively slow growth, frequent discolouration of the fish, and odours which seemed to indicate that the bacteria acted chiefly on the carbohydrates with the production of acid by-products.

The second unexpected result was the relative insignificance of the typical trimethylamine-fishy odour from the pure cultures. The odour was definite when sterile fish muscle was contaminated with fish slime or by direct contact with the cutting tables in a fish plant. A few of the cultures used, such as some of the *Proteus* strains, produced an initial fishiness which rapidly changed or was masked by other odours. The fact that an organism was a strong trimethylamine-oxide reducer in specially prepared broth is no indication that it will definitely produce a typical amine odour in fish. However, as such an odour frequently develops from fish or fish slime without being quickly masked by succeeding odours, it must be accounted for by organisms not encountered in these tests or by the activity of several organisms in combination.

The types of odours encountered conform in general with those described as prevailing successively in market fish. Elliott (1947) characterizes the odours occurring in fillets as fresh—flat—sweet—stale—putrid, which would suggest the action of the *Micrococci* and *Flavobacteria* followed by the gram negative rods.

Wood (1940) has pointed out that there appears to be a succession in the prevailing genera as fish spoils. The *Cocci* and *Flavobacteria*, which were most abundant in the fresh fish, are reduced on storage, and the gram negative rods, *Achromobacter* and *Pseudomonas*, gain the ascendancy. He also states that little is known of the actual role of these various genera in spoilage. Fellers (1926) suggests that the action of the *Micrococci* on fish is to attack the carbohydrates.

That spoilage of iced or refrigerated fillets is caused chiefly if not wholly by psychrophilic bacteria is almost a foregone conclusion. Markov (1939) found that anaerobes, *E. coli* and spore-forming aerobes play no part and that spoilage is caused mainly by the psychrophilic organisms originating in the slime, gut, skin and gills of the fish. Shewan (1944) also found that a decrease in the *Micrococci* and an increase in the rod forms, especially *Achromobacter*, as fish spoils after 6 to 14 days in storage. Aschehoug and Vesterhus (1947) found that in spoiled herring the percentages of the predominating organisms were: *Achromobacter*, 58.5; *Pseudomonas*, 33.7; *Flavobacterium*, 7.0; and *Micrococcus*, 0.8. They believed that spoilage was caused mainly by *Achromobacter*. And in our previous work (Castell, Anderson and Pivnick 1948) in attempts to correlate bacterial numbers with quality or keeping time of cod fillets it was found that the most significant relationship existed when the counts were limited to psychrophilic gram negative species.

In spite of this, it is startling to see that 250,000,000 active cells of *E. coli* can be applied to 60 sq. cm. of fish muscle surface and left two weeks at 3°C. without any obvious change. And the same may be said for a large group of other mesophilic species. This emphasizes a point which has so frequently been suggested: The organisms which are chiefly responsible for the spoilage of iced fish are those psychrophilic species which have their origin in slime and gut of the fish itself, and which are also present to a lesser extent in natural ice and in sea water.

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Relation of Bacterial Counts to Quality of Cod Fillets

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ABSTRACT

Bacterial counts are valueless as a measure of the degree of spoilage in fresh fillets.

There is a very close correlation between the number of psychrophilic gram-negative organisms on fillets and their keeping time in cold storage. This correlation degenerates into a 'general tendency' which cannot always be applied to individual samples, if the counts used include all the organisms growing on plates incubated at 25°C. Counts made on plates incubated at 37°C are of no value for estimating the keeping quality of fillets stored at low temperatures.

We know that almost all spoilage of non-oily marine fish is the result of bacterial activity. It has also been shown that the total volatile bases (Beatty and Gibbons 1937, Tanikawa 1938, Hol'mov 1937, Shewan 1939a, Paladino 1943 and Perez 1943) and more particularly the trimethylamine value (Beatty and Gibbons 1937, Beatty 1938, Lintzel and Herring 1939, Shewan 1939b, Hol'mov 1939 and Notevarp 1943) are good measures of the progress of spoilage during its earlier stages. It would be very convenient if we could find a more or less fixed relationship between the amine value, or any other useful criterion of spoilage, and the bacterial content of fish muscle. Or, to restate the relationship in the form of a question: Can we use the numbers of bacteria per gram of fish as a measure either of its present quality or its future keeping time?

It is easy to show such an apparent relationship by correlating the counts and quality during successive holding periods for a given sample of fish stored under fixed conditions. But the results from such a test need not necessarily apply to data on fish taken from many different sources and having entirely different past treatments.

Under ordinary conditions, when the number of bacteria on fish is extremely high it is highly probable that it is either of poor quality or will have a very limited keeping time. But the counts ordinarily encountered on the vast majority of our fish samples are not in this category, and before we can infer much about their quality from their bacterial counts, several other factors have to be taken into consideration.

A review of the literature shows there are two diametrically opposed schools of thought on this subject. One is represented by Hunter (1922) who concluded that it is not possible even to fix a standard bacterial count for the determination of stale or spoiled fish. The other view was taken by Reay (1935) who believed

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the bacterial count to be the best of all spoilage criteria, and that it was superior to chemical tests in detecting early stages of spoilage.

As this relationship between bacterial counts and standards of quality has either been proved or taken for granted with a wide variety of other foods, it seemed worthy of further consideration in relation to fresh fish.

Definitions of Spoilage

The problem of determining what consitutes a *spoiled* fish has never been settled. Using pure cultures, it is possible to produce a wide variety of off-odours in fish that cannot be measured by any single chemical criterion. For this reason, in some of the work outlined in this paper, especially where it involved pure cultures, the spoilage rate was estimated on the basis of organoleptic tests. Two independent observers examined the fish periodically to determine the time required for the production of the first definite spoilage odours. In most of the work, however, the amount of trimethylamine in the fish is used as a measure of spoilage. Data from many thousand tests made at this station on cod and other groundfish indicate that for the average fish with normal contamination this test is the most satisfactory.

Unless otherwise specified, the bacterial counts were made with a nutrient agar described by Dyer, Dyer and Snow (1946) and the plates incubated at 25°. Bacterial counts are always in numbers per gram of fish. Trimethylamine values indicate mgm. of trimethylamine nitrogen per 100 grams of fish.

RELATIONSHIP TO PRESENT QUALITY

The improbability of being able to infer the present quality of fillets from their bacterial count is best shown by tests made in the cutting rooms of a fish plant. Immediately prior to filleting, the muscle in the round fish is sterile or almost so. Actual tests have shown that fillets leave the cutting table a few minutes later with counts ranging from a few thousands to many millions of bacteria per gram. These organisms are picked up by the fillets during the filleting and skinning operations, and reflect the sanitary conditions of the cutting tables and the dexterity of the cutters. The trimethylamine value of the fish at this period has no relation to this newly acquired bacterial load, but is determined by the previous treatment of the round fish. This is well illustrated by the data in table I. It has also been observed that an inexperienced cutter turns out fillets with a much higher bacterial content because of his inability to prevent a gross transfer of bacteria from the surface of the round fish to the surface of the fillet.

A slightly different situation occurs when fish having different initial numbers of bacteria are held until their bacterial load reaches a fixed number. Fillets were taken aseptically from codfish and contaminated by dipping them into graded dilutions made from fish slime and fish faeces. These were stored at 3°C. Table II shows that when the bacterial counts reached approximately 10^6 per gram the trimethylamine values showed a very wide variation. In this case there is a relationship between the initial count and the subsequent amine value: the lower

the initial count, the higher the amine value when the bacterial load reached 10^6 per gram.

TABLE I. Bacterial counts (no. per g.) and trimethylamine values (mg. per 100 g.) on cod fillets immediately after filleting.

	Bacterial count	Trimethylamine value
A. Fresh fillets from fresh fish—sanitary surroundings....	32,000	1.9
B. Similar to A—but filleted under unsanitary conditions..	23,000,000	1.9
C. Fillets from round fish held 5 days at 37°—filleted under sanitary conditions.....	1,100	35
D. Similar to C, but filleted under unsanitary conditions..	25,000,000	35

A third situation occurs when counts are made on fillets that have been held for 6 or 8 days at or near freezing temperatures. During this holding period the psychrophilic organisms have an opportunity to bring about some degree of spoilage. In this case it would seem that there is some likelihood of a closer correlation between the bacterial count and the amine value. Figure 1 shows this relationship for 53 fillets. Part of these were fillets taken from retail stores at the time of their arrival from a fish plant and were subsequently held for 8

TABLE II. Trimethylamine values of fillets having different initial numbers of bacteria but held at 0° until the counts reached approximately 1,000,000 per gram.

Initial bacterial count (log. of no. per g.)	Final bacterial count (log. of no. per g.)	Storage at 0° (days)	Final trimethylamine value (mg. per 100 g.)
1.3	6.0	6	89
2.1	6.1	4	47
3.6	5.9	4	36
5.6	6.0	2	14

days at O°C. As the graph shows, there is a rough approximation between the number of bacteria and the quantity of trimethylamine. The second group were fillets aseptically removed from fish and inoculated with suspensions made from diluted slime and faeces and incubated for 8 days at 3°C. This second set of fillets differed from the commercial fillets in being considerably thinner, which might accelerate the rate of spoilage. In these, the number of bacteria initially transferred to the fish varied between 25 and 10^5 per gram. As can be seen from the figure, in this group the correlation between the bacterial count and the amine value is very much less.

INFLUENCE OF TYPE OF CONTAINER

The type of container, and especially whether or not it permits free access

of air to the surface of the fish, has some effect on the growth of bacteria. This is illustrated in the four following experiments:

(1) Six waxed cardboard containers were perforated on the sides and top, packed with fish, and stored at O°C. Fillets that were similarly contaminated

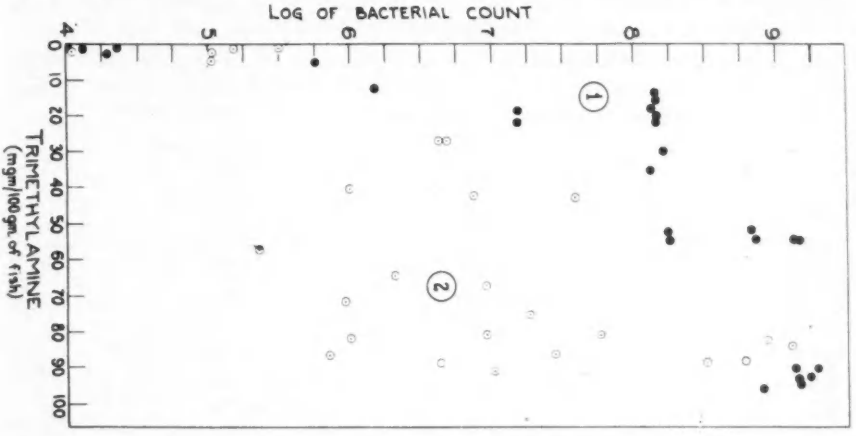

FIGURE 1. Log of bacterial counts plotted against trimethylamine value for 53 fillets which which had been held at low temperature for 8 days.
●—Commercial fillets stored 8 days at 0°C.
⊙—Sterile fillets inoculated with fish slime and feces and then held 8 days at 3°C.
(As fish with a trimethylamine value of 30-40 is already spoiled, only those samples on the left side of graph have practical significance.)

were stored in boxes that were not only not perforated but were covered with a heat-sealed plastic wrapper. Half of each set was removed from storage at 8 days and the remainder at 10 days. As can be seen in table III, there was remarkably little variation in the amine values of the sets removed at 8 days, but the average of the bacterial counts in the sealed boxes was less than half of that in those which were perforated. The results of those examined at 10 days were

TABLE III. Effect of sealing on the bacterial counts (thousands per g.) and trimethylamine values (mg. per 100 g.) of fillets packed in waxed cardboard containers, stored at 0°C. for 8 days.

	Perforated containers			Sealed containers		
	A	B	C	A	B	C
Bacteria.....	1550	1280	910	940	402	387
Trimethylamine.....	52.5	54.0	55.5	55.5	54.0	53.0

similar: the average trimethylamine values were 93.0 and 92.6 and the bacterial counts were 128×10^7 and 315×10^7 for the sealed and unsealed packages.

(2) Similarly contaminated fillets were stored in wide-mouthed glass containers. In 5 containers the tops were screwed down tightly. In others sterile glass rods were inserted in the glass jars to permit air to circulate between the glass and the fish; and the tops were left off. They were then held for 8 days at 0°C. As in all these experiments, evaporation was minimized by enclosing all the samples in a large glass container which also contained an exposed surface of water. In these tests the averages of the results were:

Containers	TMA values	Bact. counts
Capped bottles	20.8	66×10^6
Uncapped bottles	20.4	180×10^6

(3) In the tests above, the fillets were all carefully contaminated to ensure equal numbers of similar types of bacteria on the fillets. In the third set of these tests, pairs of ordinary commercial fillets were folded in half and closely packed one upon another in sterile glass beakers. Four sets were incubated for each of 5, 7 and 10 days at 2°C. As each sample was removed from storage, the top half of the top fillet and the bottom half of the bottom fillet were tested for trimethylamine and bacterial count. The results are shown in table IV. In 11 of the 12 samples, the baterial counts were greater in the partially exposed top portions of the fillets; but there was not a corresponding increase in the amine. It can also be observed that with an increase in the storage period of the fish the proportion of the bacteria on the exposed portion of the fillets becomes greater.

TABLE IV. Bacterial counts (thousands per g.) and trimethylamine values (mg. per 100 g.) for fish at the surface and at the bottom, when packed in 12 glass beakers and stored various lengths of time at 2°C.

Location of samples	Days storage at 2°C.					
	5		7		10	
	Bact.	TMA	Bact.	TMA	Bact.	TMA
Top.....	1420	19.9	60,000	43.4	65,000	41.3
Bottom.....	700	28.9	1,700	49.4	1,200	25.3
Top.....	2330	25.4	15,800	46.9	276,000	33.2
Bottom.....	280	13.0	14,200	59.0	4,000	37.3
Top.....	1700	17.4	26,000	56.5	175,000	47.3
Bottom.....	1000	22.2	1,370	31.0	12,000	37.9
Top.....	7000	37.5	8,900	57.3	111,000	34.8
Bottom.....	1440	19.0	880	37.9	12,000	62.1

(4) In a fourth set, commercial fillets were divided into two groups. The first group were stored between sheets of sterile glass with a weight on the upper glass to keep the fish firmly pressed between the sheets. The second group were laid across a series of sterile glass rods on top of a glass surface. The object of the rods was to permit air to circulate under the fillets. After 5 days' stoarge at O°C., the average trimethylamine values and plate counts were as follows:

	Exposed to air	Pressed beween glass
Bacterial counts	122,000	32,000
TMA values	1.5	2.6

RELATIONSHIP TO KEEPING QUALITY

From the results already obtained it seems obvious that there is not necessarily any relationship between the number of bacteria on a fillet and the extent to which spoilage has progressed. There is reason to believe, however, that the bacterial count may be more closely related to the future keeping quality of the fish. This also has its limitations. Bacteria are not all alike. They differ sharply in their temperature relations. Many cultures grow slowly or not at all when held below 8° or 10°C.; others thrive at freezing temperatures. Apart from temperature relationships, bacteria differ in their ability to decompose fish muscle or its ingredients. Those which are actively proteolytic or strong trimethylamine oxide reducers presumably are more important than those which have no action on these substances.

It would seem worth while, therefore, to investigate the temperature relationships of bacteria found normally on contaminated fillets, and to determine whether there is any significant difference in the fish-spoiling capacity of bacteria from different sources, before we accept the statement that 'counts are a fairly accurate criterion of future keeping quality'.

Temperature Relationships

In a series of tests, pieces of sterile fish muscle that just fit into an ordinary petri dish were inoculated with measured numbers of cells of pure cultures of bacteria and then held at 2° to 3° for twenty days. During this holding period they were examined daily for the development of spoilage odours. The results were almost startling. Inoculations consisting of millions of cells of *Escherichia coli*, *Aerobacter cloaceae*, eleven species of *Micrococcus*, *Bacillus subtilis*, *Bacillus mycoides*, *Bacillus mesentericus* and other mesophilic species, caused no perceptible change in the colour, odour or texture of the fish after 20 days' storage. However, if fish were similarly inoculated and held at 25° or 37°C. the majority spoiled very rapidly. The same thing happened when the fish stored for 20 days at 2° to 3°C. were shifted to the higher temperatures.

In contrast to this, inoculations consisting of less than 100 cells of certain unidentified species of *Proteus* and *Pseudomonas*, which were isolated from fish, brought about rapid spoilage of the fish in 5 or 6 days at 2° to 3°C. storage.

In spite of these facts, most bacterial counts on fish are made with plates

that have been incubated at 20° or 25° and some even at 37°C. If we wish to use counts from plates incubated at these higher temperatures as a measure of the keeping quality of fish stored at or near 0°C. then we should be able to find a significant correlation between counts made from plates incubated at these different temperatures.

Ten fillets were obtained from retail stores and various fish plants in the city of Halifax. By taking each one from a different source it was hoped that there

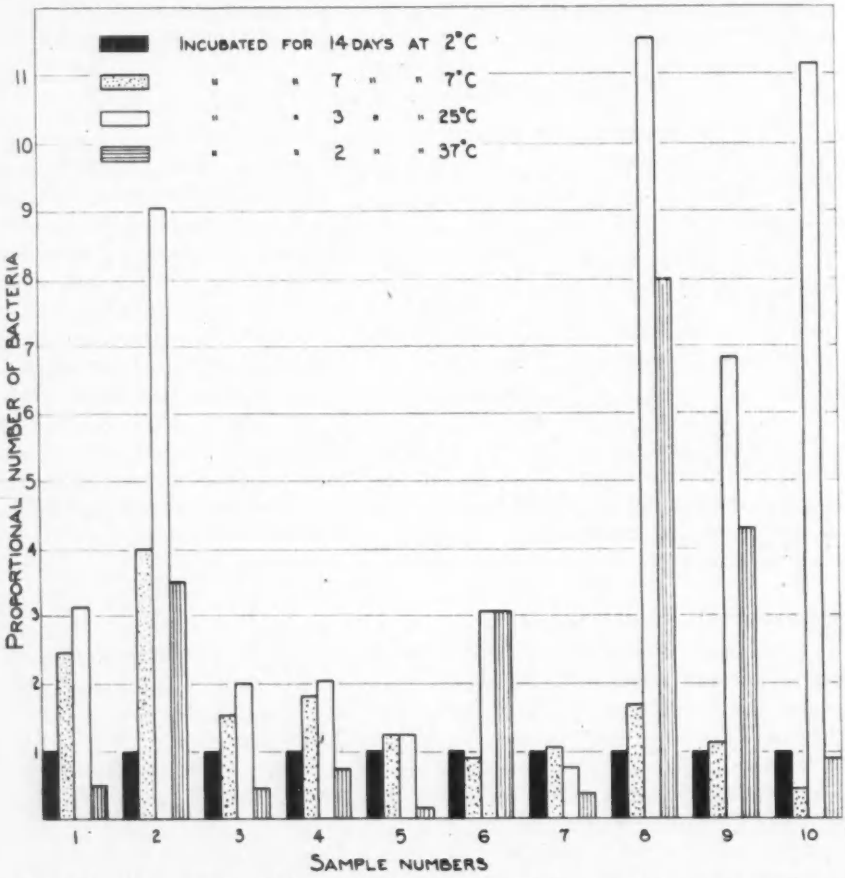

FIGURE 2. Plate counts made from ten fillets taken from different retail stores and fish plants. Plates from each fillet were incubated at 2°, 7°, 25° and 37°. In each case the count at 2° was taken as unity and the illustration shows the relative proportion of the counts at 7°, 25° and 37° compared to that at 2° for each individual fillet.

might be a wider variation in the microflora than if they had all come from one plant at the same time. Plate counts were made on each fillet from plates incubated at 2°, 7°, 25° and 37°C. for 14, 7, 3 and 2 days respectively. In figure 2

the results are tabulated so as to show the proportions between the counts at the four temperatures, using the count at 2° as the standard. It is obvious that with these 10 fillets there is no relationship between the counts at 2° and those at 25° or 37°C.

In another series, 64 fillets were taken directly from the cutting tables of a local fish plant. It was presumed there would be less variation in the flora of these fillets and, therefore, more likelihood of a closer correlation in the counts. When the counts at 25°C. were plotted against the counts at 2°C. we get the picture given in figure 3. A general trend can be discerned, especially where the

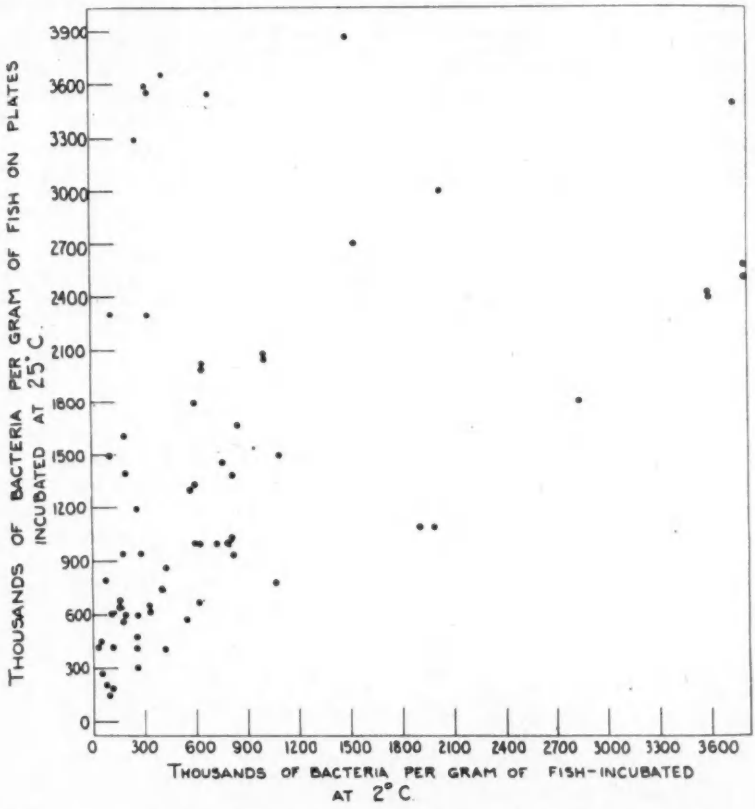

FIGURE 3. Bacterial counts on 64 cod fillets taken directly from the cutting tables of a local fish plant. In each case the counts at 25° are plotted against the counts from the same fillet when the plates were incubated at 2°C.

bacteria are less numerous. This trend, by inspection, approximated a line represented by the equation $2x = y$, where x is the count at 25° and y is the count at 2°C. But the deviations are so great and so numerous that the equation has little practical value. It is interesting to observe that with these, and also in

other similar tests, the counts at 2°C. only outnumbered those at 25°C. when the counts were excessively large; and never when the counts at either temperature were under 10^6 per gram.

Psychrophilic Types and Spoilage Rate

Using pure cultures of gram-negative psychrophilic bacteria, isolated from fish muscle, fish slime, fish faeces and sea water, an extensive series of tests was made to determine the relationship between the bacterial counts and the subsequent spoilage rate when the fish were stored at 2° to 3°C. Under these conditions, as shown by table V, there is a very definite relationship between counts and keeping time. At this temperature and with these organisms, for each tenfold reduction in the numbers of bacteria on the fillets, the keeping time was increased by approximately one day. At 0°C. the results should be even more striking. A sufficient number of tests were not done at 0°C. to state definite results. But Dyer and Dyer (1948) have found from an extensive series of tests on commercial cod fillets that fish which spoil at from 3 to 5 days at 5°C. keep from 8 to 13 days at 0°C.

Table V. Average, maximum and minimum number of days for the production of spoilage odours from fish stored at 3°C. and inoculated with measured suspensions of cells prepared from 74 cultures of gram negative, psychrophilic bacteria, of which suspensions 1 ml. was spread over 60 sq. cm. of fish surface.

Cells in inoculum (no. per ml.)	Tests made (no.)	Days before spoilage odours developed		
		Maximum	Minimum	Average
10^8 to 5×10^8	7	6	2	4.1
10^7 to 10^8	9	7	3	5.2
10^6 to 10^7	10	8	4	5.8
10^5 to 10^6	9	10	4	7.3
10^4 to 10^5	10	10	6	8.3
10^3 to 10^4	9	13	5	9.1
10^2 to 10^3	10	14	8	11.6
Under 10^2	10	<20	6	12.6+

Types of Bacteria and Rates of Spoilage

This phase of the subject is easily dealt with from a practical standpoint, but difficult to describe in scientific terms. Because of the lack of suitable chemical tests at the present stage of our knowledge, we must rely mainly on organoleptic tests for the study of pure culture spoilage of fish. Using some 60 species of psychrophilic bacteria, it was found that they produced a wide variety of off-flavours and spoilage odours. These ranged all the way from the fishy odour of trimethylamine, through sour, putrid and faecal odours. Some cultures produced ester-like, fruity or nut-like odours. Others had the typical odour of

formaldehyde or the smell associated with lactic acid. In a few instances, bacteria grew very vigorously all over the surface of the fish without producing the slightest off-odour.

In general it was found that species of the *Pseudomonas*, *Achromobacter*, *Serratia* and *Proteus*-like gram negative rods produced the most offensive types of spoilage and spoiled the fish more quickly. The *Flavobacteria*, most of the *Micrococci* and all of the *Microbacteria* produced off-flavours more slowly (table VI) and the odours were much less offensive. They rarely, if ever, produced "putrid" odours. They came more under the category of sour, musty and acid odours.

TABLE VI. Time required for the production of the first spoilage odours from fish stored at 3°C., and inoculated with measured numbers of cells of psychrophilic bacteria isolated from fish and identified to genera.

Culture	Cells in inoculum (no. $\times 10^6$)	Days before spoilage odours developed
Proteus	350	4
"	42	3
"	26	4
"	13	7
Pseudomonas	1080	1
"	34	5
"	25	6
"	12	5
"	6	6
Achromobacter	150	6
"	1200	3
"	271	3
"	72	5
Flavobacterium	250	11
"	24	7
"	13	10
"	180	13
Micrococci	390	14
"	127	10
"	303	10
"	89	17
"	26	< 20

SUMMARY AND DISCUSSION

When applied to fresh fillets, bacterial counts cannot be correlated directly with the quality of the fish. The counts may only be the result of recent contamination, while the quality is determined by the action of bacteria on the surface of the round fish as well as on the fillet. Furthermore, spoilage during the fillet stage is more closely related to the growth and activity of the bacteria than to the actual numbers present. Even if fish are held for several days or a

week at storage temperatures it is difficult to make an accurate estimate of quality from bacterial counts.

The most accurate prediction of the keeping quality of fillets can be made by determining the numbers of gram-negative psychrophilic species present. The "total count" on plates incubated at 20° or 25°C. gives a rough estimation of the keeping time, but individual results, especially when the counts are excessive, cannot be relied upon. The counts at 37°C. have no value at all in estimating the keeping time of fillets stored at or near 0°C.

These results coincide fairly closely with the suggestion of Wood (1940): "If a medium were devised which would eliminate the gram positive cocci and the *Flavobacteria*, it is considered that a much greater value could be placed on the bacterial count which would then be composed of *Achromobacter* and *Pseudomonas* which are predominant during the later stages of spoilage". If Wood's suggestion is combined with the findings of Markov (1945) the picture is completed. This latter worker came to the conclusion that spoilage in fish is due to psychrophilic bacteria which constitute the normal flora of the gut, skin and gills, and to a slight extent to certain mesophilic bacteria. He also found that anaerobes, *E. coli* and aerobic spore formers usually play no part.

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Chromatographic Analysis of the Unsaponifiable Matter of Marine Animal Oils*

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ABSTRACT

A method of separation of the unsaponifiable matter of marine animal oils has been developed, using the technique of flowing chromatography. Four fractions were obtained by the successive use of light petroleum, methylene chloride, ethyl ether, and methanol with a column of alumina. On the basis of these studies, the hypothesis is proposed that the substances occurring in any one fraction are of similar chemical structure.

The fatty acid composition of natural fats and fatty oils has long been studied and in recent years notably by Hilditch (1940) and co-workers, among many others. The composition of the unsaponifiable matter ("unsap") which accompanies the triglycerides has received less attention, probably because the amount of unsap in fat is usually small. However, in some marine animal oils there may be a considerable amount of unsap. In some oils the presence of certain components, notably vitamins A and D, in the unsap is of great significance commercially.

Hydrocarbons are present in traces in many marine animal oils and constitute a very considerable portion of the oil in some cases. One or more monohydroxy alcohols are universally present, either esterified or in the free state. Cholesterol, vitamins A and D, some pigments, and fatty alcohols fall in this class. Dihydroxy alcohols, as represented by the glyceryl ethers, form a major proportion of the unsap of some oils. A method of chromatographic analysis is presented for the separation of these groups from the non-saponifiable fraction of marine animal oils.

Many naturally occurring mixtures have been examined by this method. They are discussed in several recent books (Zechmeister and Cholnoky 1941, Strain 1942). The unsaps of fatty oils are among those natural mixtures to have received attention. In some cases the separation of a valuable component was the reason for the investigation as, for instance, vitamin A (Karrer *et al.* 1931, Heilbron *et al.* 1932, Holmes *et al.* 1932-33), and vitamin D (Brockmann 1936). Chromatography has been frequently advocated as a means of separating a vitamin from interfering substances in preparation for its determination. Vitamin A in egg yolk has been separated from xanthophylls (Mann 1943). Vitamin D has been separated from vitamin A and pigments (Marcussen 1939; Ewing *et al.* 1943;

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De Witt and Sullivan 1946). The separation of pigments in intensely coloured marine oils has been accomplished frequently (Burkhardt *et al.* 1934; Bailey 1937, 1938; Mori and Sato 1939). The separation of pigments from plant sources has been described many times (Strain 1942). In addition, as contributions to knowledge, separation of the unsaps of various marine oils has been attempted (Channon *et al.* 1934; Thorbjarnarson *et al.* 1935; Nakamiya 1939; Ruiz 1942).

In some of the investigations mentioned above, the classical method of Tswett was used. A solution of the mixture under study was passed through a vertical column of powdered adsorbent which, after development with more solvent, was cut into portions either empirically or by appearance when coloured bands were present. The separated portions of the column was then extracted with an appropriate solvent and the extracts were analyzed.

In other studies the later method of the "flowing chromatogram" was used. After development of the column with a suitable solvent, another solvent was passed through the column which eluted only the lower band(s) of adsorbed material, resulting in a separation of the mixture originally added to the column. Little or no hint was given as to the basis of selection of suitable solvents. Trappe (1940) gave the clue to this selection. He listed a series of solvents in what he termed an "elutropic series", wherein each stated solvent had a stronger eluting action than any following it regardless of the adsorbent in use. This list simplified the selection of suitable solvents for a flowing chromatogram. A recent paper by Strain (1946) states that the selection is not always so simple as indicated by Trappe.

EXPERIMENTAL

The first problem attacked in this investigation was the selection of an adsorbent suitable for the adsorption of vitamin A. Solutions of fish liver oil unsap containing vitamin A were made up in light petroleum and were passed through columns, each containing a different one of seventy-one powdered chemicals taken directly from their containers. Passage of vitamin A through the column into the eluate was followed by examination with ultraviolet light, in which vitamin A exhibited a characteristic greenish-yellow fluorescence. Successive portions of the eluates were examined for their vitamin A content by the Carr-Price reaction, using a Rosenheim-Schüster tintometer. (An Evelyn photoelectric colorimeter was used in all subsequent determinations.) Most of the chemicals allowed unimpeded passage of vitamin A, the initial eluate showing the same concentration of vitamin as the original solution. A few caused marked destruction of the vitamin, and several showed favourable adsorptive properties. The detailed results of this preliminary investigation have been published (Swain 1941).

Since alumina was found to be satisfactory and was readily available commercially, it was selected as the adsorbent for the present studies. In the earlier work to be described below, 40- to 80-mesh activated alumina (Aluminum Ore Co. of America, Grade A) was ground to pass an 80-mesh sieve, and was used without further treatment. Pressure from a cylinder of nitrogen was necessary

to force solvents through the column. In later work grade F1 alumina from the same source was ground to pass an 80-mesh sieve and the portion retained by a 150-mesh sieve was used. This portion was heated at 180°C. for several hours before using. Solvents passed readily through columns of such alumina at atmospheric pressure. In both cases the alumina was packed firmly with a glass rod to a depth of 10 cm. in a glass tube 1 cm. inside diameter. A plug of cotton at the constricted lower end prevented loss of alumina from the column.

Dogfish (*Squalus suckleyi*) liver oil, a valuable source of vitamin A during the war years, was used to find a series of solvents suitable for the separation of the components of fish oil unsaponifiable matter. The unsap was obtained from the oil by the S.P.A. method (Society of Public Analysts Committee 1933), but using four extractions with ether instead of three (Swain 1944). This unsap was dissolved in light petroleum (Skellysolve F) and passed through the column (alumina A, -80 mesh), which was developed by passage of a further 100 ml. of solvent. A yellow band of pigment was usually about two-thirds down the column and did not move during the latter part of the development with light petroleum. Examination with ultraviolet light (G.E. Purple X 250 watt lamp) showed a band of vitamin A fluorescence immediately above the pigment band (figure 1).

In so treating the unsaps of the oils from 30 separate dogfish livers, less than 3% of the unsap was found in the light petroleum eluate in every case. The average amount eluted was 1.7%. The colourless liquid recovered from these eluates gave no precipitate when its acetone solution was saturated with dry hydrogen chloride indicating the absence of squalene (Tsujimoto 1920).

Various solvents and mixtures of solvents were used in an attempt to elute vitamin A from the column without the simultaneous escape of other components of the unsap. Mixtures were unsatisfactory mainly because of the difficulty of maintaining uniformity of composition during recovery for later use. Recourse to solvents in the elutropic series of Trappe (1940) showed that methanol, acetone, ethyl ether and chloroform were all too strong in their eluting ability, since all or most of the unsap escaped from a column washed with one of them. Acetone was useless for another reason also, namely its dimerisation during passage through the column (Zechmeister and Cholnoky 1941, p. 5), resulting in adulteration of the unsap recovered from it. Cyclohexane, a weak eluting solvent, was used to follow light petroleum in the elution of an unsap, but 500 ml. removed only a trace of material from the column.

Benzene (CP) eluted the vitamin A and the pigment, which showed a yellow-green and a reddish-brown fluorescence respectively in ultraviolet light. Progress of vitamin A through the column was determined by measuring the amount present in successive 50-ml. portions of the benzene eluate. Results are shown in table I in which is also given the weight of unsap eluted in each eluate portion. Passage of vitamin A paralleled passage of other components, all dwindling to negligible proportions. This benzene eluate also contained all the cholesterol in the unsap as was demonstrated in the following experiment. One half of a solu-

tion of unsap in light petroleum was passed through a column which was developed with more of the same solvent. Benzene (225 ml.) was then passed through the column. Cholesterol was determined in the benzene eluate and in the other half of the unsap by Ireland's modification (1941) of the Liebermann-Burchard reaction, using an Evelyn photoelectric colorimeter with 540-mμ filter and omitting the preliminary purification procedure. The unsap portion (328.5 mg.) con-

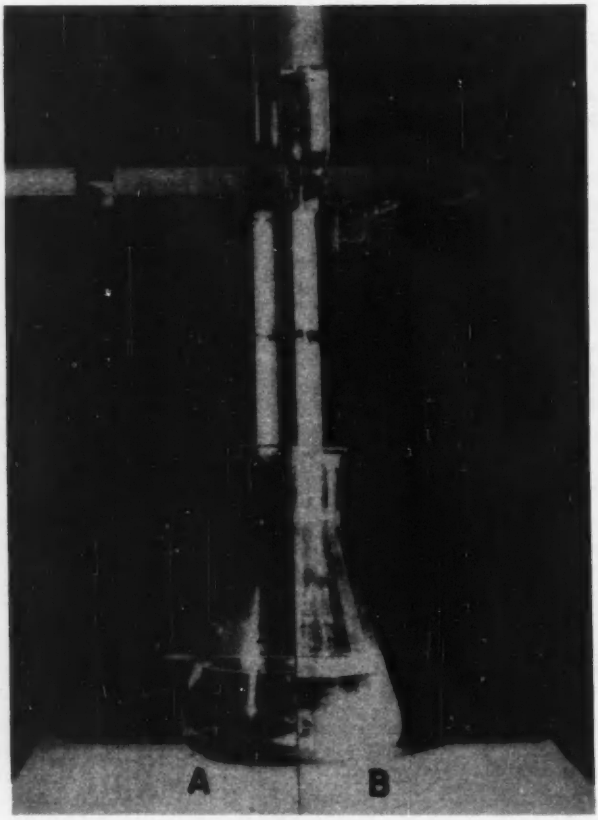

FIGURE 1. Alumina column containing dogfish liver oil unsap, after development with light petroleum. A. Photographed in electric light. B. Photographed in ultraviolet light.

tained 4.32 mg. cholesterol and the benzene eluate residue (45.3 mg.) contained 4.25 mg. cholesterol. That is, essentially all the cholesterol present appeared in the benzene eluate.

Benzene seemed to be a satisfactory solvent, although a rather large volume was required to elute all of the vitamin A. The addition of 2% of ether to benzene increased its power of elution but was not used for long because of the

above-mentioned difficulty of recovery of mixed solvent. Benzene eluted an average of 10.7% (range 3.7 to 28.6%) of unsaps obtained from the thirty dogfish liver oils mentioned above. The material eluted by this solvent contained pigment, vitamin A, cholesterol, and an unidentified liquid.

In an attempt to separate further these substances the residues from several benzene eluates were combined and passed through a column in light petroleum

TABLE I. Vitamin A content of successive benzene eluates from a dogfish liver oil unsap adsorbed on alumina (Alorco, grade A)

Eluate (50-ml. portions)	Residue (mg.)	Vitamin A (U.S.P. units)
1	1.7	80*
2	1.1	30*
3	0.9	40*
4	12.5	262
5	8.7	236
6	3.3	54
7	1.0	23
8	0.3	10

*Approximate values only.

solution. All the material was adsorbed. After development, the column was washed with carbon tetrachloride, an eluant weaker than benzene (Trappe 1940). The solvent was collected in 50-ml. portions in each of which was determined the total weight of dissolved material, the weight of cholesterol and the number of U.S.P. units of vitamin A. The results are shown in figure 2. It is evident that the greatest weight of the benzene eluate fraction, a liquid, was the most readily eluted, and that cholesterol was the most firmly adsorbed. However, no clean-cut separation of the components of this mixture was accomplished.

Passage of ethyl ether (U.S.P., stored over acidified ferrous sulphate and distilled before use) through a column which had been eluted with benzene washed out the greatest proportion of the unsap, averaging 79.5% (range 60.3 to 91.4%) of the unsaps of 24 of the above dogfish liver oils. This material was demonstrated to be glyceryl ethers, earlier reported present in the liver oil from dogfish under the name *Squalus wakiyae* (Toyama 1924), which was later stated to be synonymous with *S. sucklii* (Tsujimoto 1935). Chimyl alcohol was crystallized from its ether or light petroleum solution at −10°C. The crystals were readily separated by centrifuging, providing the cooled solution was first well stirred to enable packing of the crystals. Otherwise much of the mother liquor remained trapped between the crystal plates. After several recrystallizations these crystals amounted to 14-23% of the ether eluate in the oils examined. They melted at 61.5-62°C. (chimyl alcohol 62-62.5°C., Knight 1930) and had a hydroxyl value 10.86 by the method of Malm *et al.* (1935) (chimyl alcohol 10.75). The mother liquors from such crystallizations consisted of a yellow liquid, whose bromination in ethyl ether solution at −2°C. yielded from 0.1 to 2.6% of white precipitate

in those from the oils examined (calculated in each case from the weight of the original eluate residue). Transfer of the ether-soluble portion to light petroleum resulted in a second white precipitate of the same order of quantity. The total gain in weight of the mother liquors on bromination corresponded closely to the calculated value of 46.7% for selachyl alcohol. This eluate therefore contained chimyl alcohol, selachyl alcohol and a small proportion of highly unsaturated

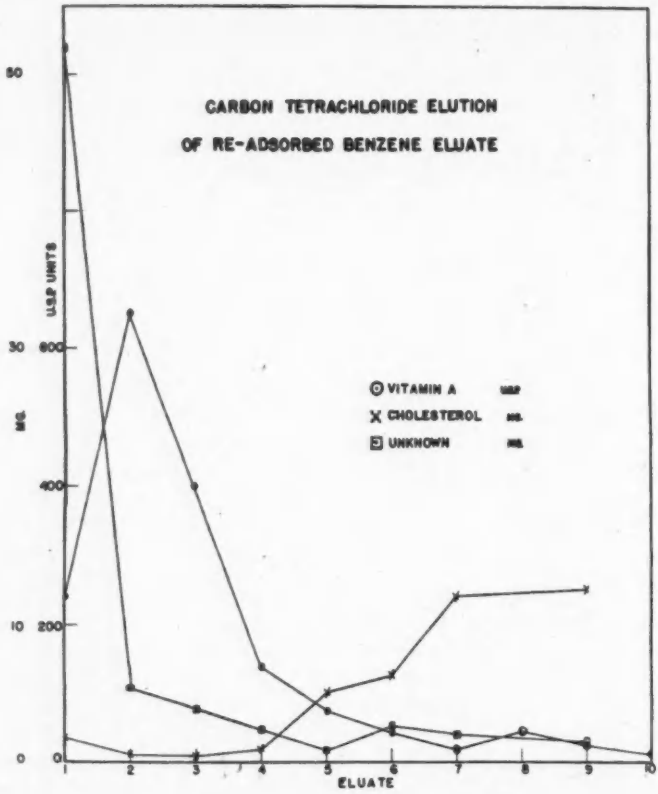

FIGURE 2. Composition of the carbon tetrachloride eluates obtained by chromatographing the benzene eluate residue of a dogfish liver oil unsap, both on alumina.

material, presumably the unsaturated glyceryl ethers reported by Toyama and Takahasi (1939).

Finally, passage of methanol (technical, distilled) through the column eluted 0.5 to 6.1% (average 2.4%) of the unsap of the above dogfish liver oils. This material is unstable. It was liquid when recovered from a column operated in the dark with nitrogen pressure, but semi-solid and only partially soluble in light petroleum when recovered from an unsap passed through a column in the daylight with compressed air.

This series of solvents—light petroleum, benzene, ethyl ether, methanol—thus separated the components of dogfish liver oil unsap into four groups of compounds: (a) A colourless unidentified liquid melting between −6° and 0°C., which was not squalene; (b) pigment, vitamin A, cholesterol, and an unidentified liquid; (c) glyceryl ethers, including chimyl alcohol, selachyl alcohol, and a small proportion of more highly unsaturated material, presumably also glyceryl ethers; (d) a liquid not identified. The absorption curves of these four groups of compounds are shown in figure 3. They were determined on composite samples through the kindness of Dr. G. Halpern of The Canadian Fishing Company, using

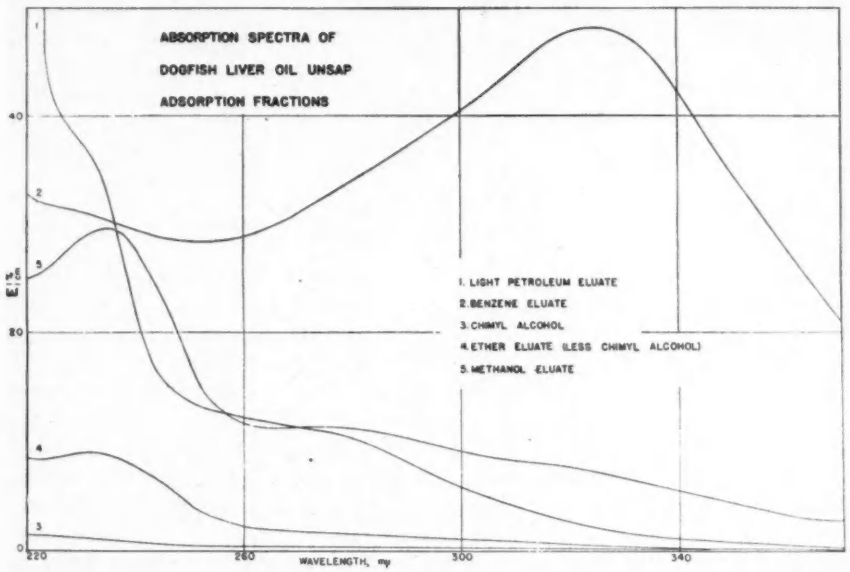

FIGURE 3. Absorption spectra of the eluate residues obtained by chromatographing dogfish liver oil unsap on alumina.

a Beckman D.U. spectrophotometer. With the number of oils examined, no connection was noted between the composition of the unsap and the size or sex of the fish from which it was obtained.

A fresh supply of alumina from the same source, labelled grade F1 but claimed to be identical with grade A, and freshly activated before use, was not as satisfactory as the grade A product (probably because of the activation) in that vitamin A did not escape readily from the column with benzene. Methylene chloride (technical) was found to be sufficiently stronger in eluting ability to remove vitamin A almost completely from the column. Its only disadvantage lay in the fact that it also caused elution of the glyceryl ethers, which were in eluate fractions escaping from the column after most of the vitamin A had been removed. (See table II.)

TABLE II. Fractions separated chromatographically from a dogfish liver oil unsap, using methylene chloride as the eluting agent. C represents cholesterol; F indicates the fluorescence of vitamin A.

Solvent	Volume (ml.)	Eluate residue				Appearance
		Sample 1		Sample 2		
		(mg).	(%)	(mg.)	(%)	
Light petroleum....	100	2.0	0.4	2.1	0.4	Colourless liquid
Methylene chloride	50	0.9		0.6		Yellow liquid, F
	50	1.0		1.3		" " "
	50	3.2		4.8		" " F, C
	50	11.3		9.6		Yellow solid, F, C
	50	0.2		1.0		C
	50	0	3.0	1.1	3.0	
	50	1.9		5.5		Yellow liquid
	50	18.3		17.8		" "
	50	34.5		46.1		" semi-solid
	50	30.2	15.4	34.0	18.1	" waxy solid
Ethyl ether.....	150	323.7		334.0		" solid
	150	14.6	61.7	20.9	61.6	" "
Methanol.....	100	25.5	4.7	28.9	5.0	" liquid

The procedure finally adopted was as follows:

Alumina (Alorco grade F1), 80- to 150-mesh size, was used to pack the column. A light petroleum solution of unsap of about 25% concentration was passed into the column, which was developed with light petroleum until the colour band was sharp and without movement during passage of solvent, and until dissolved material no longer was present in the eluate fractions; 150 ml. normally sufficed. Methylene chloride was then added to the column. When it had penetrated to the colour band, the latter appeared red with the ultraviolet light source used, and the receiver was then changed. The solution was collected in 50-ml. portions from which the solvent was removed and the residues weighed. These decreased successively to a very small quantity and then began to increase. The vitamin A fluorescence was very faint in eluates following those containing only a small amount of solute. The increasing weights were due to elution of the glyceryl ethers. Ether was then passed through until 150-ml. portions of the eluate contained little or no residue. This solvent eluted the glyceryl ethers much more readily. Finally, passage of 100 ml. of methanol through the column eluted the remainder of the adsorbed material.

In table III are given the results of three separate analyses of samples of unsap prepared from one oil, showing the replicability of the method. In table IV are the results obtained from samples of commercial dogfish liver oils produced in British Columbia.

TABLE III. Chromatographic analysis of samples of a dogfish liver oil unsap (Alumina F1). Bracketed values are considered to be glyceryl ethers and are included in the ether eluate totals.

Solvent	Volume (ml.)	1 Eluate residue		2 Eluate residue		3 Eluate residue	
		(mg.)	(%)	(mg.)	(%)	(mg.)	(%)
Light petroleum.....	50	7.0		8.1		8.3	
	50	1.3		0.9		0.7	
	50	0.7		0		0.1	
		9.0	2.1	9.0	2.1	9.1	2.0
Methylene chloride.....	100	12.7		15.0		14.9	
	50	16.0		19.2		13.8	
	50	5.0		2.3		6.3	
	50	2.3		0.1		2.0	
		36.0	8.4	36.6	8.6	37.0	8.3
		(14.9)		(13.6)		(11.6)	
Ethyl ether.....	150	285.7		282.1		283.0	
	150	41.8		54.8		50.8	
	50	5.5		1.7		6.2	
	50	3.2		2.8		4.2	
	50	2.9		2.4		2.3	
	50					2.8	
		354.0	82.6	357.4	83.6	360.9	81.3
Methanol.....	100	27.4	6.4	27.2	6.4	26.0	5.9
Recovery.....			99.5		100.7		97.5

TABLE IV. Chromatographic analysis of the unsaps from commercial British Columbia dogfish liver oils (Alumina F1)

Oil no.	Unsap (%)	Eluate residue (%)			
		Light pet.	Methylene chloride	Ethyl ether	Methanol
1	27.5	0.6	4.1	78.5	10.8
2	22.2	1.4	7.2	76.3	12.1
3	20.6	0.9	8.3	69.1	12.3
4	25.0	0.8	14.7	87.1	4.4
5	23.8	3.1	11.3	78.6	7.6
6	22.3	1.7	8.9	78.9	5.7
7	20.6	1.7	7.2	79.5	8.8
8	23.2	1.7	5.8	84.1	3.3

During the development of this method of analysis, it was applied to the unsaps of oils from other marine sources (Swain and McKercher 1945). The details of procedure varied with the stage of development of the method. In all cases a given solvent was used until the residue in a given volume of eluate became negligible in amount. The results are shown in table V.

TABLE V. Chromatographic analysis of the unsaps of several marine animal oils.

Unsap from oil of	Alumina	Light petroleum		Second solvent		Ethyl ether		Methanol	
		(ml.)	(% eluted)	(ml.)	(% eluted)	(ml.)	(% eluted)	(ml.)	(% eluted)
Halibut liver.....	A	150	1.0	2100°	91.8	300	9.3	100	4.9
Lingcod liver.....	A	100	1.7	900*	78.6	300	12.3	100	6.5
	A	100	1.6	900*	78.7	300	12.2	100	5.1
	F1	100	1.3	500†	70.0	300	18.8	100	5.7
	F1	100	1.7	500†	69.1	300	17.7	100	7.5
Basking shark liver..	F1	225	98.1	350†	5.4	150	1.3	100	1.6
	F1	225	93.6	350†	4.7	150	1.5	100	1.7
Soup-fin shark liver..	F1	600	2.8	600†	40.8	300	11.9	900	36.3
Mackerel shark liver.	F1	200	1.7	550†	77.7	150	4.9	100	12.1
Sperm whale blubber.	F1	250	0.7	700†	88.9	150	1.0	100	5.9
Rat-fish liver.....	F1	150	1.2	300†	29.7	450	68.6	100	1.7

°Benzene. *2% ether in benzene. †Methylene chloride.

The unsap from the liver oil of halibut is known to be mainly cholesterol (Haines and Drummond 1933). This was demonstrated with a sample of oil containing 8.5% unsap. The portion of unsap readily soluble in light petroleum was added in that solvent to a column of alumina. After development with the same solvent, the remainder of the sample of unsap was dissolved in benzene and added to the column. Most of the sample was eluted from the column by benzene, and the melting point of the residues from the later fractions showed them to be almost pure cholesterol. The vitamin A in the unsap also appeared in these benzene eluates.

A sample of liver oil from lingcod (*Ophiodon elongatus*) contained 12.2% unsap, a deep yellow liquid in which were suspended a few crystals at room temperature. Chromatographic analysis of the unsap yielded a pale yellow solid in the light petroleum eluate, with an intense band of pigment remaining in the column. Two per cent ether-in-benzene mixture eluted most of the unsap. In a later analysis methylene chloride eluted slightly less of the unsap. In this experiment the solid eluted by light petroleum was tested with the Liebermann-Burchard reagent and was shown to be free from cholesterol.

The unsap of the liver oil from the basking shark (*Cetorhinus maximus*) is known to be largely the hydrocarbon squalene (Tsujimoto 1917; André and Canal

1928). The unsap from a sample of this oil (52.2% of the oil, and a colourless mobile liquid) passed through a column of alumina almost completely in the light petroleum in which it was dissolved. The eluate residue remained liquid at −25°C. Passage of hydrogen chloride through a cold acetone solution of the residue produced a copious white precipitate with m.p. 120-1°C., a test for squalene (Tsujimoto 1920). This experiment offered confirmation of the earlier work and showed that the hydrocarbon squalene passes through the column as rapidly as does the solvent light petroleum, since practically all of it appeared in the first two eluates.

The writer has seen no description of the composition of the unsap of soup-fin shark liver oil (*Galeorhinus galeus*) other than relative to its high content of vitamin A. The oil from a composite sample representing over 100 cans of livers contained 5.7% unsap, a viscous yellow liquid at room temperature. The small portion of it which was eluted by light petroleum was a white solid. The fractions eluted by methylene chloride were solid, probably because of the cholesterol content. All other fractions eluted from the column were liquid, the proportion eluted by methanol being uniquely large among the oils examined.

The liver oil from a mackerel shark (*Isurus nasus*) (Swain and Sidaway 1944) contained 7.9% of an unsap which by chromatographic analysis was quite similar in composition to that of halibut liver oil.

A sample of oil prepared commercially from the blubber of the sperm whale (*Physeter macrocephalus*) led to interesting results. The unsap of this oil is known to consist mainly of fatty alcohols (Toyama 1927), and amounted to 35.8% of the oil examined. Almost none of it was eluted by light petroleum in its chromatographic analysis and no band of pigment was evident. Elution of the column with methylene chloride washed out nearly all the unsap, from which it follows that this fraction must therefore contain the fatty alcohols. The appearance of successive eluates implied that the liquid oleyl alcohol was eluted somewhat ahead of the solid cetyl and stearyl alcohols.

The liver oil from the rat-fish (*Hydrolagus colliei*) is known to contain glyceryl ethers (Lovern 1937). The unsap from a sample of oil, containing 33.6% unsap, gave results on chromatographic analysis similar to those obtained with dogfish liver oil unsap.

These data reduce the universality of a method for the determination of unsap proposed by Sylvester *et al.* (1945). In this method the ether extract prepared by the S.P.A. method is acidified without previous washing. The resulting ether solution is water-washed to remove the inorganic acid and dried. The solution of unsap and free fatty acids is passed through a column of aluminal which is then washed with more ether. The unsap is claimed to pass through and the free fatty acids to be adsorbed. However, in all the oils reported above ethyl ether did not elute all of the unsap. In the case of mackerel shark liver oil and of soup-fin shark liver oil, a very considerable portion of the unsap remained in the column after elution with this solvent. The unsap as determined by this method will therefore be lacking by that amount.

On the other hand, some free fatty acids are eluted by ethyl ether. Dogfish liver oil fatty acids (0.5 g.) prepared by acidifying the soap solution remaining after extraction of an unsap were passed in light petroleum solution through a column of alumina which was then developed with more light petroleum until 150 ml. eluate was obtained. This eluate left no residue on evaporation. Passage of 150 ml. methylene chloride eluted 0.8% of the fatty acids, and subsequent passage of 300 ml. ethyl ether eluted 42.3%. The last 50-ml. portion still contained 5% of the total material eluted. The unsap as determined by the method of Sylvester *et al.* may therefore contain free fatty acids, and may not include all the components of the unsap of the oil.

DISCUSSION

The results obtained in this series of experiments suggest that substances of similar chemical structure are eluted by a given solvent, with some partial degree of separation because of variations of that chemical structure. It is recognized that hydrocarbons are eluted by light petroleum (Fitelson 1943). This was demonstrated with the unsap from basking shark liver oil, from which squalene was shown to be separated in a column by light petroleum.

The substances recognized to be present in the methylene chloride eluate included vitamin A, cholesterol and fatty alcohols. These are all monohydroxy in composition. A partial separation of vitamin A and cholesterol was evident by visual examination of the eluate residues together with measurement of the vitamin A. This separation was demonstrated by the use of carbon tetrachloride as eluting solvent, a solvent below methylene chloride in Trappe's series. The alcohols in sperm whale oil unsap were partially separated as evidenced by the difference in solidity of successive eluate residues.

Ethyl ether eluted the glyceryl ethers from the unsaps of dogfish and of ratfish liver oils. Chimyl alcohol and selachyl alcohol were recognized to be present in dogfish liver oil unsap and also material sufficiently unsaturated to be precipitated by bromination in ether solution. In this eluate, compounds of varying C-atom content and of varying unsaturation shared the property of being dihydroxy in nature, which is possibly the dominant characteristic of the ethyl ether eluate.

The material eluted by methanol was in most cases too small in amount for examination. It seemed to be unstable in nature.

SUMMARY

The unsaps of marine animal oils are separable into simpler mixtures of compounds by the method of the flowing chromatogram. It was shown that hydrocarbons such as squalene are not adsorbed from light petroleum by alumina. Of the other substances present, monohydroxy substances are least readily adsorbed being eluted by benzene or, more readily, by methylene chloride; and dihydroxy substances are eluted with difficulty by methylene chloride but readily with ethyl

ether. Other substances, not eluted by ether, are removed from the column by passage of methanol.

The unsap of dogfish liver oil was shown to contain only a very small proportion of hydrocarbons but rather to be largely glyceryl ethers. The composition of the unsaps of oils from individual dogfish livers showed quantitative differences. The unsap of a rat-fish liver oil was of qualitatively similar composition.

The unsaps from oils of other fishes and from the sperm whale were found to differ in composition very considerably. The unsap from basking shark liver oil was largely unadsorbed, and is known to be mainly hydrocarbon in nature; the unsaps from sperm whale oil and halibut liver oil were mostly eluted by benzene or methylene chloride and are known to be principally monohydroxy substances.

A published method for the determination of unsaponifiable matter was shown to be open to two possible errors, namely, incomplete elution of unsap from alumina by ethyl ether, and incomplete adsorption of fatty acids from ethyl ether by alumina.

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